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# Quantifying nitrous oxide production pathways in wastewater treatment systems using isotope technology – a critical review

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## Abstract

Nitrous oxide ($N_2O$) is an important greenhouse gas and an ozone-depleting substance which can be emitted from wastewater treatment systems (WWTS) causing significant environmental impacts. Understanding the $N_2O$ production pathways and their contribution to total emissions is the key to effective mitigation. Isotope technology is a promising method that has been applied to WWTS for quantifying the $N_2O$ production pathways. Within the scope of WWTS, this article reviews the current status of different isotope approaches, including both natural abundance and labelled isotope approaches, to $N_2O$ production pathways quantification. It identifies the limitations and potential problems with these approaches, as well as improvement opportunities. We conclude that, while the capabilities of isotope technology have been largely recognized, the quantification of $N_2O$ production pathways with isotope technology in WWTS require further improvement, particularly in relation to its accuracy and reliability.

***Keywords**: nitrous oxide, pathways, quantification, isotope, site preference, wastewater treatment*

## 1. Introduction

Nitrous oxide ($N_2O$) is the third most abundant greenhouse gas (GHG) in atmosphere and also the most significant sink for stratospheric ozone layer. Since 1970, global $N_2O$ emissions have increased by 43%, and at present, $N_2O$ makes up 6.2% of total anthropogenic GHG emissions. Nitrous oxide has a long atmospheric lifetime (116 years compared to 12 years for $CH_4$)(Prather et al.) and a large global warming potential (310 times that of $CO_2$) (Edenhofer et al. 2014). Apart from its potential effect on climate, $N_2O$ can also react with atomic oxygen to produce nitric oxide (NO), which damages the stratosphere. A comparison study with other ozone-depleting substances suggested that $N_2O$ is currently the most significant anthropogenic ozone-depleting substance being emitted (Ravishankara et al. 2009), and will have increased ozone depletion potential in the next hundred years (Revell et al. 2015).

It is now well recognised that wastewater treatment systems (WWTS) are important sources of $N_2O$ to the atmosphere (Kampschreur et al. 2009b, Law et al. 2012b). Emissions from wastewater treatment have steadily increased during recent decades reaching 108 $MtCO_2$ eq in 2010 comprising 3.4% of the global $N_2O$ emission budget (Edenhofer et al. 2014). Motivated by the strong desire to mitigate emissions, considerable research has been undertaken to understand the impact of various operational parameters on these pathways (Kampschreur et al. 2009b, Law et al. 2012b). Studies initially focused on the effects of operational variables on $N_2O$ emission using a 'black box' approach. With this approach, $NO_2^-$ ,dissolved oxygen (DO), chemical oxygen demand (COD) and pH have been investigated and recognized as the most relevant parameters affecting $N_2O$ production in WWTS (Kampschreur et al. 2009b). The black box approach can

generally support mitigation strategies development for a given system; however, it is often difficult to extrapolate findings from one system to another, due to contradictory observations. For example, low DO concentrations have been reported to stimulate $N_2O$ emission in conventional nitrifying and denitrifying systems (Kampschreur et al. 2008), which was contradicted by the observation that a lower DO concentration decreased $N_2O$ emissions in a partial nitrification (nitritation) anammox system (Kampschreur et al. 2009a). Similarly, a higher pH resulted in a higher $N_2O$ production in a partial nitrification system (Law et al. 2011) while reducing $N_2O$ production in a denitrification system (Hanaki et al. 1992). Inconsistent results were also reported for nitrite. A positive relationship between $NO_2^-$ concentration and $N_2O$ production was reported for a laboratory nitrifying reactor (Peng et al. 2015b), a full-scale wastewater treatment plant (WWTP) (Foley et al. 2010) and also a full-scale nitritation-anammox reactor (Kampschreur et al. 2009a). In contrast, an inhibitory effect of $NO_2^-$ on $N_2O$ production was found in partial nitrification reactors (Law et al. 2013, Wang et al. 2016). Clearly, the 'black box' approach can lead to unreliable or system-specific conclusions, which hinder the general applicability of research outcomes. Therefore, process-based pathway knowledge is required to better understand the effects of the operating conditions on $N_2O$ production and to ensure the transferability of outcomes from one system to another. In addition to supporting generic mitigation strategy development, the $N_2O$ pathway knowledge also facilitates the development of $N_2O$ emission as a monitoring tool in WWTS. Produced mostly from biological nitrogen removal pathways in WWTS, the $N_2O$ emission data contains valuable information about the activity of AOB. $N_2O$ emission data can provide early warning for nitrification failure (Burgess et al. 2002, Butler et al. 2009), and potentially monitor the nitrification process (Butler et al. 2009, Wunderlin et al. 2013b).

To gain insights into the $N_2O$ production pathway knowledge and establish more reliable mitigation strategies, the ambiguities surrounding the dynamics of pathways relative contributions must be addressed. In WWTS, $N_2O$ is mostly produced biologically in nitrification and denitrification via a number of pathways including the nitrifier denitrification pathway and the hydroxylamine ($NH_2OH$) oxidation pathway employed by ammonia-oxidizing bacteria, and the heterotrophic denitrification pathway employed by heterotrophic denitrifiers (Ni and Yuan 2015). In addition, $N_2O$ can also be formed chemically from $NH_2OH$ and $NO_2^-$ via abiotic reactions (Harper Jr et al. 2015, Soler-Jofra et al. 2016, Terada et al. 2017). Different $N_2O$ production pathways often occur simultaneously. Each pathway is regulated differently by environmental factors (Law et al. 2013, Peng et al. 2014), and thus needs to be analysed individually by the relative contribution to total $N_2O$ emission. For example, DO suppresses denitrification while it promotes nitrification, thus having different impact on $N_2O$ production by different pathways. Consequently, the dynamics of pathways relative contributions is critical to the mitigation. Although $N_2O$ production pathways have been investigated in a large number of pure culture studies, the pathway dynamics, i.e. the relative contributions from each pathway are not well-understood.

In the past decade, isotope techniques have been applied increasingly to WWTS to study $N_2O$ production pathways. Isotope technology has been used to unravel previously convoluted pathways, as such the $N_2O$ production pathways themselves can be effectively quantified without affecting their magnitude. However, the application of isotope techniques to WWTS is still in early stages. In particular there is still much room for improvement in the very promising site-preference analysis application, which was introduced as recently as 2011 (Toyoda et al. 2011a) to WWTS. This paper reviews the natural abundance and labelled isotope approaches that have been used to

92 investigate the relative contributions of various $N_2O$ production pathways in WWTS. The review
93 is conducted from a critical perspective, focusing on the principles and developments, and also
94 identifying the limitations.

95 **2. Current knowledge about $N_2O$ production pathways**

96 In WWTS, $N_2O$ is mostly produced by biological nitrogen removal processes, which
97 consist of nitrification and denitrification. There are three main microbial pathways and two groups
98 of bacteria involved in $N_2O$ formation (Figure 1). During nitrification, $N_2O$ is produced by
99 autotrophic AOB via the $NH_2OH$ oxidation pathway (Kampschreur et al. 2009b, Law et al. 2012b).
100 During denitrification, $N_2O$ is emitted by both autotrophic AOB via the nitrifier denitrification
101 pathway, and heterotrophic denitrifiers via the heterotrophic denitrification pathway
102 (Kampschreur et al. 2009b, Law et al. 2012b). In addition, it has been realized more recently that
103 the abiotic/biotic N-nitrosation hybrid $N_2O$ production pathway can also contribute significantly
104 to the $N_2O$ emission under some extreme circumstances when $NO_2^-$ and $NH_2OH$ concentrations
105 are high (Soler-Jofra et al. 2016, Terada et al. 2017).

106 During nitrification, $NH_2OH$ is produced as the intermediate when AOB oxidize ammonia
107 ($NH_3$) to nitrite ($NO_2^-$). In the $NH_2OH$ oxidation pathway, $N_2O$ is formed as a by-product during
108 the oxidation of $NH_2OH$ to $NO_2^-$ by AOB (Hooper 1968, Ritchie and Nicholas 1972) (Figure 2A).
109 In vitro stoichiometry and metabolism studies of $NH_2OH$ oxidation catalysed by hydroxylamine
110 oxidoreductase (HAO) in *Nitrosomonas* revealed the formation of a nitrogenous intermediate
111 which is probably nitroxyl (HNO) (Anderson 1964, Falcone et al. 1963). HNO reacts with another
112 $NH_2OH$ and 2 $e^-$ to form hyponitrite (ONNO) which decomposes to $N_2O$ (Yamazaki et al. 2014).
113 NO reduction by NO reductase and/or cyt c554 may also be involved in the $N_2O$ production
114 process (Beaumont et al. 2004, Upadhyay et al. 2006). In addition, a recent study by Caranto et al

(2016) demonstrated a direct production of $N_2O$ from $NH_2OH$ by cyt P460 (a c-type heme of HAO) under anaerobic conditions. This finding along with other studies suggested that when the environmental condition is switched from aerobic to anaerobic, $N_2O$ can still be produced from the $NH_2OH$ oxidation pathway (Caranto et al. 2016, Law et al. 2012a). Furthermore, it has been hypothesized that $N_2O$ may actually be the main product of $NH_2OH$ oxidation under aerobic conditions and the observed $NO_2^-$ is a by-product of NO oxidation (White and Lehnert 2016).

The nitrifier denitrification pathway is a type of denitrification carried out by autotrophic AOB reducing $NO_2^-$ to $N_2O$ (Poth and Focht 1985). Nitrifier denitrification is generally believed to occur preferably under oxygen-limiting conditions (Poth and Focht 1985, Wrage et al. 2001). During nitrifier denitrification, $NO_2^-$ is firstly reduced to nitric oxide (NO) by a periplasmic copper-containing nitrite reductase (nirK) (Garbeva et al. 2007, Hooper 1968) and/or an unidentified nitrite reductase (Kozlowski et al. 2014). Nitric oxide is then further reduced to $N_2O$, catalysed by a membrane-bound nitric oxide reductase (NorB) (Casciotti and Ward 2005, Garbeva et al. 2007). Nitric oxide reduction is the major process for $N_2O$ production during both nitrifier denitrification and heterotrophic denitrification. $N_2O$ reductase ($N_2OR$) is not found in AOB, making $N_2O$ the end product in the nitrifier denitrification pathway.

In the heterotrophic denitrification pathway, heterotrophic denitrifiers carry out stepwise reduction of $NO_3^-$ or $NO_2^-$ to $N_2$ via $N_2O$ as an obligate intermediate. Each reaction in this stepwise reduction process has been verified through numerous enzymology studies of nitrate reductases (NaR), nitrite reductases (NiR), nitric reductases (NOR) and nitrous oxide reductases ($N_2OR$) (Hochstein and Tomlinson 1988). Similar to nitrifier denitrification, most $N_2O$ emitted during heterotrophic denitrification is from the reduction of nitric oxide catalysed by nitric oxide reductase (Hochstein and Tomlinson 1988). During nitric oxide reduction, one NO molecule binds

to the active Fe center of nitric oxide reductase (NOR). Hyponitrite (ONNO) is formed as an intermediate when another NOR bounded NO molecule binds (Watmough et al. 2009). Hyponitrite will then easily decompose to produce $N_2O$ (Figure 2B). Although $N_2O$ is the end product in the nitrifier denitrification pathway, $N_2O$ can be reduced to $N_2$ by $N_2OR$. Thus the heterotrophic denitrification pathway can act as both a source and sink for $N_2O$ (Robertson and tiedje 1987).

The N-nitrosation hybrid $N_2O$ production pathway is a coupled biotic-abiotic process where $N_2O$ is produced from the interaction of $NO_2^-$ and microbially-produced nitrification intermediate $NH_2OH$ (Zhu-Barker et al. 2015). The reaction can be written as (Schreiber et al. 2012): ($NH_2OH + HNO_2 \rightarrow N_2O + 2H_2O$). However, the reaction is often masked by biological $N_2O$ production (Heil et al. 2014). This pathway was confirmed in WWTS very recently in a $^{15}N$-labeling study (Terada et al. 2017). The two N atoms in $N_2O$ originate from an N atom from $NO_2^-$ and an N atom from $NH_2OH$ (Frame et al. 2017, Stieglmeier et al. 2014, Terada et al. 2017). Recent studies showed that $N_2O$ produced via the N-nitrosation hybrid $N_2O$ production pathway can be significant (Harper Jr et al. 2015, Soler-Jofra et al. 2016, Terada et al. 2017). For example, in a partial nitrification system (Single reactor High Activity ammonium Removal over Nitrite, SHARON), the N-nitrosation hybrid $N_2O$ production pathway was estimated to contribute to one third of the total $N_2O$ emission (Soler-Jofra et al. 2016). This was likely because of the relatively high concentrations of $NH_2OH$ (resulting from a high $NH_3$ oxidation rate) and $HNO_2$. Since the $NH_2OH$ and $HNO_2$ concentrations are normally very low in conventional WWTS, $N_2O$ produced via the N-nitrosation hybrid $N_2O$ production pathway is likely insignificant, and has indeed often been neglected in pathway quantification analysis conducted so far. This assumption remains to be verified in future experimental studies.

Activated sludge in a WWTS comprises a complex mixture of microorganisms and metabolic pathways. Pure culture studies may not necessarily represent how production pathways are regulated in mixed cultures under WWTS conditions. Similarly, results obtained from mixed culture studies in soil or marine environments may not be applicable for WWTS, since the WWTS environment is a substantially different environment, engineered to achieve a high level of nitrogen removal from wastewater. A targeted approach is therefore required to study the individual N2O pathways and mitigation strategies in WWTS. On the other hand, the highly engineered nature of the design and operational conditions of WWTS provides a promising opportunity to mitigating N2O emission once the pathways and the key influencing conditions are identified (Law et al. 2012b).

## 3. $N_2O$ production pathways quantification with conventional natural abundance analysis

### 3.1 Stable isotope foundations

Elements exhibit multiple isotopes which have a unique number of neutrons; some are radioactive while others are stable. Unlike radioactive isotopes, stable isotopes do not decay. Natural nitrogen and oxygen are composed of several common stable isotopes, namely $^{14}N$, $^{15}N$ and $^{16}O$, $^{17}O$, $^{18}O$, respectively. The lighter isotopes are in far greater abundance than the heavier ones, at 99.64% for $^{14}N$ and 99.76% for $^{16}O$, versus 0.36% for $^{15}N$, 0.04% for $^{17}O$ and 0.20% for $^{18}O$ (de Laeter et al. 2003). The abundance of $^{15}N$ and $^{18}O$ in emitted $N_2O$ has been widely applied to study $N_2O$ pathways. It was believed that the $^{17}O$ changes in a certain correlation with the change of $^{18}O$ and does not provide addition information (Röckmann et al. 2001); thus $^{17}O$ is not studied in WWTS. However, it was later proven that $^{17}O$ in $N_2O$ molecules has a small but measurable deviation of that correlation and can be a tracer of $N_2O$ source (Mukotaka et al. 2013). With the

development of measurement techniques (Dyckmans et al. 2015, Mukotaka et al. 2013) and accelerating applications of $^{17}O$ in other environmental field (Costa et al. 2011, Michalski et al. 2004, Tsunogai et al. 2011), it will likely be introduced into WWTS in the coming years.

### 3.2 Stable isotope abundance measurement and denotation

The abundance of elemental nitrogen and oxygen isotope can be directly and simultaneously measured by a conventional dual-inlet isotope ratio mass spectrometry (IRMS) equipped with a triple-Faraday-cup collector system (Kim and Craig 1993, Tanaka et al. 1995). The modification enables the simultaneous measurement of three ion beams of 44, 45 and 46, and thus nitrogen ($^{15}N$) and oxygen ($^{18}O$) isotopes in $N_2O$ can be determined by the analysis of the 45 to 44 and 46 to 44 mass ratios, respectively. In the analysis, since having the same molecular masses as $N_2O$ molecules, interferences of $N_2{}^{17}O$ (45) and $CO_2$ (44) needs to be addressed. The abundance of $^{17}O$ can be calculated by the mass-dependent fractionation relationship between $^{17}O$ and $^{18}O$ (Assonov and de Groot 2009, Röckmann et al. 2003); and the interference of $CO_2$ can be prevented by chemical traps and GC prior to IRMS measurement (Tanaka et al. 1995). In the past decade, laser spectroscopy has been developed as an alternative to the widely-used IRMS method to determine nitrogen isotope abundance (Uehara et al. 2003, Waechter and Sigrist 2007). It has the advantage that no sample preparation is required; thus continuous measurement under field conditions may be achieved (Waechter et al. 2008). However, the IRMS method is generally more accurate than laser spectroscopy (Mohn et al. 2014).

Measurement of differences between the standard absolute isotope abundance and the sample isotope abundance provides a reliable and convenient way to express the isotope abundance level. Although the direct measurement of the absolute isotope abundance value is possible, it requires a complicated and highly accurate calibration process and its accuracy can suffer from a

number of effects thus analytically challenging. In contrast, the measurement of differences between the standard abundance and sample abundance is usually easier and do not suffer from the same type of effects (Vogl et al. 2016). Thus, to better interpret the isotope abundance, differential analysis is generally used (Equation 1) (Craig 1961, McKinney et al. 1950).

$$\delta = \frac{R_{sample} - R_{standard}}{R_{standard}} \quad (1)$$

Where δ is the isotope ratio relative to a standard. $R_{sample}$ and $R_{standard}$ are the absolute isotope ratios of the sample and standard, respectively (Craig 1961, McKinney et al. 1950). The standards normally used for nitrogen and oxygen are atmospheric $N_2$ and Vienna Standards Mean Ocean Water (VSMOW) respectively. The δ value is conventionally expressed in "per mil" (parts per thousand, ‰). A detailed guideline for the expression of stable isotope ratio has been published by Coplen (2011).

### 3.3 Isotope mixing and fractionation

Isotope mixing and fractionation are the fundamental principles that govern isotope circulation in the biosphere: mixing unites isotopes while fractionation separates them (Fry 2006). Isotope mixing happens when two or more sources contribute to the end product. The isotope mixing model quantifies the relative contributions from the sources to the mixture. Analyzing isotope mixing with a mass balance model can determine the contributions of sources (Phillips and Koch 2002).

When two sources with known, substantially different isotopic compositions are mixed, the isotopic composition of the mixture indicates their relative proportions. Their proportions can be calculated through mass balance analysis as illustrated by Equation 2, 3 (Fry 2006).

$$m_{\text{mixture}} = m_1 + m_2 \quad (2)$$

$$\delta_{\text{mixture}} * m_{\text{mixture}} = \delta_1 * m_1 + \delta_2 * m_2 \quad (3)$$

where $\delta_{mixture}$, $\delta_1$, and $\delta_2$ are the isotope values of mixture, source 1, and source 2, respectively. $m_1$, $m_2$,and $m_{mixture}$ are the masses of source 1, source 2, and mixture, respectively.

If the fraction ($f_1$) for source 1 and the fraction ($f_2$) for source 2 is used (Equation 4, 5),

$$f_1 = m_1 / m_{mixture} \quad (4)$$

$$f_2 = m_2 / m_{mixture} = 1 - f_1 \quad (5)$$

the fraction of source 1 ($f_1$), namely the contribution from source 1, can be obtained as:

$$f_1 = ( \delta_{mixture} - \delta_2 )/( \delta_1 - \delta_2 ) \quad (6)$$

In contrast to the mixing process, isotope fractionation separates isotopes. Differences between isotopes of the same element are so subtle that can be regarded as identical in general reactions. However, during kinetic reactions, when bonds are forged or broken, the slight difference in physical and chemical properties can lead to fractionation, altering the composition of natural isotopes in products (Philip Wilson Rundel 1988). This variation in composition serves as the basis to monitor and trace the nitrogen conversion pathways during $N_2O$ production.

Microbial nitrogen conversions leading to $N_2O$ production can result in unique isotopic fractionation, thus making it possible to distinguish the sources of the emitted $N_2O$. Nitrogen conversions in microorganisms are catalyzed by enzymes. In most cases, the enzymatic reactions preferentially convert lighter isotopes, resulting in products isotopically lighter than in reactants. This is called kinetic isotope effects (Fry 2006). In addition, inverse kinetic isotope effects which lead to heavier isotopes more depleted in reactant also occurs in some enzymatic reactions, e.g., the bacterial nitrite oxidation (Casciotti 2009). Since distinctive enzymatic discriminations may fractionate $^{15}N$ (or $^{18}O$) into specific different isotope abundance levels in the products, the resulting $^{15}N$ (or $^{18}O$) abundance levels can be a possible indicator of the origin and pathway of the emitted $N_2O$.

### 3.4 Enrichment factors

To describe the extent of fractionations, enrichment factor ($\varepsilon$) of the product relative to the substrate in a closed system is defined by using the 'Rayleigh' equation (Equation 7) (Mariotti et al. 1981):

$$\varepsilon = \frac{10^3 * \ln \frac{10^{-3}\delta X_S + 1}{10^{-3}\delta X_{S,0} + 1}}{\ln f} \quad (7)$$

Where $\delta X_{S,0}$ , $\delta X_S$ stand for the initial and residual isotopic composition of a substrate at a reaction time, and $f$ is the residual substrate at the reaction time represented as a fraction of the initial amount of the substrate.

As for the mathematical approximation ( $\ln(1+x) \approx x$; $\ln \frac{1+x}{1+y} \approx x - y$ ), most $\varepsilon$ can be described by a simplified version of Equation 7, written as in Equation 8. The simplified version is effective for $|\varepsilon| < 20$ ‰ and $\delta X_{S,0}$ value close to zero. Inaccuracy increases towards the limits, hence Equation 7 is recommended in extreme cases (Mariotti et al. 1981).

$$\varepsilon = \frac{\delta X_S - \delta X_{S,0}}{\ln f} \quad (8)$$

To express the enrichment factor as a function of the isotopic composition of the accumulating product and initial substrate, Equation 8 can be combined with the isotope balance equation (Equation 9) giving rise to Equation 10. Thus the enrichment factor ($\varepsilon$) can be determined as the slope of the relationship of $-(f \ln f)/(1-f)$ to $\delta X_P$ during the reaction (Sutka et al. 2008, Sutka et al. 2006).

$$f * \delta X_S + (1-f)\delta X_P = \delta X_{S,0} \quad (9)$$

$$\delta X_P = \delta X_{S,0} - \varepsilon \frac{f \ln f}{1-f} \quad (10)$$

In WWTS, the product ($N_2O$) concentration is much lower than the substrate ($NH_4^+$, $NO_2^-$ or $NO_3^-$) concentration and $\varepsilon$ values for nitrification and denitrification are usually much smaller

than 1000 so that isotopic composition of substrate can be regarded constant. Therefore, a simplified enrichment factor relation is obtained in Equation 11. This simplified enrichment factor used for WWTS (Toyoda et al. 2011a), is opposite to the net isotope effect factor ($\Delta\delta X$, a difference in the isotope values between the product and substrate) as written in Equation 12 (Koba et al. 2009, Mohn et al. 2012).

$$\delta X_P = \delta X_S + \varepsilon \quad (11)$$

$$\Delta\delta X = \delta X_S - \delta X_P \quad (12)$$

### 3.5 Understanding $N_2O$ production pathways in WWTS with natural abundance analysis and enrichment factors

Measurement of the $\delta^{15}N$-$N_2O$ value in emitted $N_2O$ can qualitatively estimate the contribution from nitrification and denitrification in WWTS. Generally, studies have shown that the $\delta^{15}N$-$N_2O$ value from the anoxic or anaerobic tank favoring denitrification is higher than that from the aerobic tank favoring nitrification (Toyoda et al. 2009) (Table 1). The difference in $\delta^{15}N$-$N_2O$ values between nitrification and denitrification is large enough to qualitatively estimate the contribution of nitrification and denitrification processes of emitted $N_2O$. Within one WWTS, a higher $\delta^{15}N$ value in emitted $N_2O$ represents a greater contribution from denitrification while a lower value reflects a greater contribution from nitrification. However, unlike the $\delta^{15}N$ value, the $\delta^{18}O$ value is not well constrained. A few studies have shown that the $\delta^{18}O$-$N_2O$ value is not significantly different for various sources (Toyoda et al. 2011a). Thus the naturally abundant $\delta^{18}O$-$N_2O$ value is not an effective method to study the contribution of $N_2O$ production processes.

However, for studies related to more than one WWTS, the $\delta^{15}N$-$N_2O$ value analysis can hardly be used to qualitatively study the origin of $N_2O$, because $\delta^{15}N$-$N_2O$ is dependent on the substrate $\delta^{15}N$-$NH_4^+$ values and fractionations. If $\delta^{15}N$-$NH_4^+$ varies between two WWTSs, the

297 comparison of $\delta^{15}N$ values of the $N_2O$ emitted from the two WWTS cannot indicate the
298 fractionations relevant to nitrogen conversion process. $\delta^{15}N\text{-}NH_4^+$ likely varies between WWTSs,
299 considering spatial variations of $\delta^{15}N\text{-}NH_4^+$ (Pérez et al. 2006, Tilsner et al. 2003). In contrast,
300 enrichment factors may be used to compare the extents of fractionation between WWTSs.

301 It was proposed that $\delta^{15}N$ and $\delta^{18}O$ enrichment factors may reflect specific microbial
302 nitrogen conversion processes in environment (Casciotti 2009, Kim and Craig 1990). Assuming
303 that each WWTS receives wastewater that has a unique isotopic composition within a short period
304 of time, the $\delta^{15}N$ and $\delta^{18}O$ enrichment factors can be suitable indicators of the origin of the $N_2O$
305 emitted. However, studies have reported significant variations of $\delta^{15}N$ and $\delta^{18}O$ enrichment factors
306 even for a single $N_2O$ production pathway, as summarized in Table 2. For the $NH_2OH$ oxidation
307 pathway, the $\varepsilon(^{15}N)$ obtained range from -10.5‰ to 5.7‰. Similarly, inconsistent $\delta^{15}N$ enrichment
308 factors were measured in the heterotrophic denitrification pathway, varying from -36.7‰ to
309 19.5‰. Even larger variations were captured for the nitrifier denitrification pathway where the
310 $\delta^{15}N$ enrichments varied from -56.9‰ to 29.2‰. These studies indicate that there is not a universal
311 enrichment factor for each of $N_2O$ production pathways.

312 **3.6 Limitations of conventional natural abundance analysis**

313 To date, the $\delta^{15}N$ and $\delta^{18}O$ natural abundance values and enrichment factors have not been
314 used to quantify the contribution of nitrification and denitrification to $N_2O$ production in any
315 environment. Quantification can only be made possible if a set of universal enrichment factors are
316 available for the $N_2O$ production pathways. Enrichment factors represent the extent of overall
317 fractionation. If the overall fractionation is solely dependent on the microbial $N_2O$ production
318 pathways, it can be possible that a set of universal enrichment factors exist for each $N_2O$ production
319 pathway. However, the overall isotope fractionation measured during the biological nitrogen

320 removal process is not only determined by the enzymatic fractionation, but also affected by other
321 non-enzymatic processes irrelevant to nitrogen conversion pathway such as, the cellular diffusion
322 rate, oxygen atom exchange, and evaporation (Toyoda et al. 2015) which explains why the
323 enrichment factors obtained in a same pathway are not consistent; and also why it is not practical
324 to use enrichment factors to quantify $N_2O$ production pathway contributions. These effects make
325 the quantification with conventional natural abundance analysis inherently inclusive. Thus it has
326 only been used in WWTS to analyze the sources (nitrification and denitrification) qualitatively.

327 **4. $N_2O$ production pathways quantification based on site-preference (SP) analysis**

328 **4.1 Site-preference concepts and measurement**

329 Site-preference analysis was initially applied to WWTS in 2011. It is a promising
330 technology for quantifying $N_2O$ production pathways based on natural isotope abundance (Toyoda
331 et al. 2011a). To understand site-preference, some background concepts need to be introduced.

332 Isotopocules are molecular species with differences in either the number or positions of
333 isotopic atoms. More specifically, molecular species with the same number of isotopic atoms but
334 at different positions are called isotopomers (Coplen 2011). Because of the asymmetric linear
335 structure (N-N-O) of $N_2O$, there are eight $N_2O$ isotopocules. Among them, $^{14}N^{14}N^{16}O$, $^{14}N^{15}N^{16}O$,
336 $^{15}N^{14}N^{16}O$, and $^{14}N^{14}N^{18}O$ are the most abundant isotopocules in nature. $^{14}N^{15}N^{16}O$ and $^{15}N^{14}N^{16}O$
337 are two isotopomers with the $^{15}N$ atom in the central and outer position, respectively. Subject to
338 isotopic fractionations, the distribution of $^{15}N$ in central and outer positions is different. The
339 difference of $^{15}N$ abundance in central and outer positions is called site-preference (SP, Equation
340 13). To avoid confusion, the conventional $\delta^{15}N$ value including central and outer positions is
341 named $\delta^{15}N_{bulk}$. The relationship between $\delta^{15}N$ bulk value, SP value and $\delta^{15}N_{\alpha}$, $\delta^{15}N_{\beta}$ values is

described in Equations 13 and 14. Subscripts α and β stand for central and outer position of N atom.

$$SP = \delta^{15}N_{\alpha} - \delta^{15}N_{\beta} \quad (13)$$

$$\delta^{15}N_{bulk} = \delta^{15}N = (\delta^{15}N_{\alpha} + \delta^{15}N_{\beta})/2 \quad (14)$$

Toyoda and Yoshida (1999) and Brenninkmeijer and Röckmann (1999) independently developed a method using fragment-ion analysis with IRMS to determine the intramolecular distribution of $^{15}N$ atom in $N_2O$ isotopomers. In this approach, the fragment ions ($NO^+$, m/z 30 and 31) produced during the electron ionization of $N_2O$ possess the N atoms in the central position of $N_2O$ molecules while the molecule ions ($N_2O^+$, m/z 44, 45 and 46) contain the general isotope ratio information ($^{15}N/^{14}N$, $^{18}O/^{16}O$). Thus, the measurement of both fragment ions and molecule ions enables the quantification of isotope ratios for the central and outer nitrogen atoms in the $N_2O$ molecule. The rearrangement reaction that certain fractions of outer N atom exchanged into the fragment ion needs to be corrected in quantification and the fraction is dependent on the mass spectrometer used (Frame and Casciotti 2010, Toyoda and Yoshida 1999, Toyoda et al. 2015). In addition to the conventional IRMS approach, which is widely used for WWTS (Ostrom et al. 2007, Sutka et al. 2006, Toyoda et al. 2011a, Tumendelger et al. 2016), the laser spectroscopy has been developed for the $N_2O$ isotopomer measurement in more recent years (Mohn et al. 2012, Uehara et al. 2003, Waechter et al. 2008, Waechter and Sigrist 2007). It is inherent of laser spectroscopy to differentiate between $^{14}N^{15}N^{16}O$ and $^{15}N^{14}N^{16}O$. Compared to IRMS, the laser spectroscopy may be better suited for $N_2O$ isotopomer measurement as assessed in an isotopomer analysis study (Mohn et al. 2014). With the precision and sensitivity enhancement by employing quantum cascade laser absorption spectrometry (QCLAS) (Waechter et al. 2008), the laser spectrometry has now been applied more to WWTS to measure isotopomers (Harris et al. 2015, Heil et al. 2014,

Peng et al. 2014, Wunderlin et al. 2013a). More recently, real-time isotopomer measurement was achieved by Mohn et al. (2012) which can be useful for online quantification and monitoring of $N_2O$ production pathway (Wunderlin et al. 2013a). However, both the mass spectrometer and the laser spectroscopy measurement methods have difficulties in accuracy which will be further discussed in Section 4.6.

### 4.2 Site-preference fractionations

Unlike the $\delta^{15}N_{bulk}$ value which can be affected by numerous factors, the site-preference value of $N_2O$ reflects mostly the corresponding $N_2O$ production processes, and can thus be used to trace the pathways for $N_2O$ formation.

For $N_2O$ produced during biological nitrogen removal in WWTS, the formation of the enzyme-bound molecule and the cleavage of the N-O bond results in fractionation of $^{15}N$ into α and β positions (Yoshida and Toyoda 2000). As mentioned in Section 2, $N_2O$ is produced from NO reduction and $NH_2OH$ oxidation via three $N_2O$ production pathways. During these processes, hyponitrite is formed before being decomposed to produce $N_2O$ (Anderson 1963).

The hyponitrite formed during $NH_2OH$ oxidation, most likely breaks the O atom bond in the first $NH_2OH$ molecule which binds HAO (Yamazaki et al. 2014). Thus the N atom in the first-bound $NH_2OH$ molecule will be at the β position of the $N_2O$ formed from hyponitrite decomposition, while the N atom in the second-bound $NH_2OH$ molecule will be at the α position (Figure 3A). In this case, the SP value is mostly dependent on the molecule-binding sequence. Generally, in kinetic reactions the lighter isotopes react faster (Fry 2006). For instance, the $^{14}N$-$NH_2OH$ molecule preferentially binds to the catalytic centre of HAO as compared with $^{15}N$-$NH_2OH$ molecule, which leads to more $^{14}N$ at the β position, and $^{15}N$ at the α position. The

387 expected positive SP value is consistent with the measured values reported in literatures (Frame
388 and Casciotti 2010, Sutka et al. 2006).

389 NO reduction is likely the step that determines the SP value of $N_2O$ in denitrification
390 (Yamazaki et al. 2014). The fractionation mechanism for NO reduction (Figure 3B) is different
391 from that for $NH_2OH$ oxidation since nitric reductase (NOR) has a binuclear centre containing two
392 ferric atoms (Watmough et al. 2009). Two NO molecules bind to the ferric atoms of the NOR
393 simultaneously to form the hyponitrite to engender $N_2O$ (Watmough et al. 2009). Unlike the
394 hyponitrites formed during $NH_2OH$ oxidation, the hyponitrites formed during NO reduction are
395 uniform in structure. The uniformity leaves little chance for isotopic preference. $N_2O$ engendered
396 from NO reduction therefore has a SP close to zero (Yamazaki et al. 2014). Nevertheless, NOR
397 might have a specific preference during bond rupture which yields a slightly negative SP value in
398 the heterotrophic denitrification pathway (Frame and Casciotti 2010, Sutka et al. 2006, Wunderlin
399 et al. 2013a, Yamazaki et al. 2014).

400 The SP value of $N_2O$ from the nitrifier denitrification pathway is likely similar to the value
401 from the heterotrophic denitrification pathway, because the fractionation mechanisms of the two
402 pathways might be the same. Previous studies suggested $N_2O$ production related nitrite reductase
403 (NirK) and nitric oxide reductase (NorB) in AOB is phylogenetically close to that from
404 denitrifying bacteria (Garbeva et al. 2007, Kondo et al. 2012). In some studies, very similar SP
405 values were obtained for $N_2O$ emitted from the nitrifier denitrification pathway and for $N_2O$ from
406 the heterotrophic denitrification pathway (Sutka et al. 2006, Sutka et al. 2004), while in other
407 studies significantly different values were reported (Frame and Casciotti 2010).

408 The SP value is believed to be independent of substrate isotopic compositions and bulk
409 $\delta^{15}N$ fractionations (Sutka et al. 2006), which makes it universally applicable. Different substrate

410 isotopic compositions and varying bulk $\delta^{15}N$ fractionations caused by processes irrelevant to
411 nitrogen conversion pathway (e.g., substrate diffusion) can result in divergent enrichment of $^{14}N$
412 or $^{15}N$ atoms during the nitrogen conversions. But the variations of enrichment will hardly affect
413 the SP value (Sutka et al. 2006). The substrate isotopic composition will vary with the absolute
414 number of hyponitrite molecules. However, the SP value reflects the difference between $^{15}N^{14}N^{16}O$
415 and $^{14}N^{15}N^{16}O$, which is only affected by the formation and cleavage of the hyponitrite consisting
416 of one $^{14}N$ atom and one $^{15}N$ atom. Notwithstanding the effects of varying substrate isotopic
417 compositions and processes irrelevant to nitrogen conversion pathway, the site-preference value
418 can serve as a tool to determine the relative contributions of each pathway to the total $N_2O$
419 emission.

420 **4.3 SP signature values for different $N_2O$ production pathways**

421 $N_2O$ produced from different pathways display distinct and relatively constant SP values
422 (Table 3), known as pathway signature values. For the $NH_2OH$ oxidation pathway, SP values
423 directly measured from pure culture and mixed culture studies are generally from 28.4‰ to 36.6‰
424 while for the nitrifier denitrification pathway SP values range from -1.7‰ to 0.1‰. Although SP
425 value of the nitrifier denitrification pathway estimated from a mixing model based on SP and $\delta^{18}O$-
426 $N_2O$ measurements is significantly lower at -10.7‰ (Frame and Casciotti 2010), the oxygen
427 exchange issue (Section 5) may discredit this approach. Similar to the SP values of the nitrifier
428 denitrification pathway, SP values measured in pure culture experiments for the heterotrophic
429 denitrification pathway are mostly from -5.1‰ to -0.5‰. Despite the fact that abiotic reactions
430 have not been considered in SP-based pathway quantifications yet, the SP values for abiotic
431 reactions (including the N-nitrosation hybrid $N_2O$ production pathway) have been obtained. Like
432 the other pathways, the SP values for abiotic reactions are also relatively constant, ranging from

29.5‰ to 35.2‰. SP values measured for each of the $N_2O$ production pathway have been summarized in Table 3, which also includes a short description of each of the experiments used for the SP determination. The SP signature values of $N_2O$ production pathways obtained in different studies were relatively constant which indicate that the SP value, unlike enrichment factors, is mainly determined by the $N_2O$ production processes per se (Sutka et al. 2006, Toyoda et al. 2015). In addition, different bacterial species share similar SP signature values for the same pathway (Sutka et al. 2006). This similarity makes the SP values of $N_2O$ production pathways universally applicable when attributing $N_2O$ pathway sources. Although SP signature values were initially determined via pure culture studies, numerous SP studies have since been done to quantify $N_2O$ production pathways in various environments (Fujii et al. 2013, Koba et al. 2009, Peng et al. 2014, Yamagishi et al. 2007).

The signature values for each $N_2O$ production pathway form the basis of applying SP measurements to investigate $N_2O$ production pathways (Frame and Casciotti 2010, Sutka et al. 2006, Toyoda et al. 2005, Wunderlin et al. 2013a). Thus, accurate and reliable determination of SP signature values is of great importance. By obtaining SP signature values of $N_2O$ production pathways from different pure culture bacteria (*N. europaea, N. multiformis, M. trichosporium, P. chlororaphis and P. aureofaciens*), the study of Sutka et al (2006) confirmed that the SP value can be used to determine the origin of emitted $N_2O$ and is independent of isotopic fractionation. Wunderlin et al (2013a) measured the SP signature values in WWTS for the first time. The signature values determined were later used in other WWTS $N_2O$ quantification studies (Peng et al. 2014). These signature value studies contributed to the isotope technology development significantly, although still with issues existed. More importantly, the current, widely used

455 signature values for the $NH_2OH$ oxidation pathway and the nitrifier-denitrification pathway are
456 not without questions.

457 The methodology commonly used for obtaining SP signature values is the substrate
458 separation method which supplies substrate for a single pathway in order to eliminate the other
459 pathways. Namely, to determine the SP signature value for the $NH_2OH$ oxidation pathway, the
460 only nitrogenous substrate provided in experiments is $NH_2OH$. The SP signature values were
461 firstly determined through pure culture studies, and then through mixed bacterial cultures in
462 activated sludge, to better apply the SP method in WWTS. However, the SP signature value
463 obtained for the $NH_2OH$ oxidation pathway with the substrate separation method is likely affected
464 by the nitrifier denitrification pathway. In the pure culture study reported in Sutka et al. (2006),
465 cell suspensions of *N. europaea*, *N. multiformis* and *M. trichosporium,* respectively, were prepared
466 and preconditioned to remove all $NH_4^+$, $NO_2^-$ and $NH_2OH$. In the mixed culture study (Wunderlin
467 et al. 2013a), the activated sludge was collected from an aerobic reactor of a pilot scale municipal
468 wastewater treatment plant and was continuously aerated to remove ambient $NH_4^+$. Experiments
469 were started by adding $NH_2OH$ as a pulse to each prepared bacterial suspension or activated sludge
470 in the presence of oxygen. Since neither nitrite nor nitrate was added, the SP value of the $N_2O$
471 emitted from the cell suspensions was regarded as the $NH_2OH$ oxidation pathway's signature value
472 in the pure culture study. The assumption is that the $N_2O$ emitted was solely produced via the
473 $NH_2OH$ oxidation pathway. However, this assumption might not be valid. After $NH_2OH$ is added
474 to AOB, $NO_2^-$ can be produced during $NH_2OH$ oxidation. The concentration of nitrite was not
475 monitored in the pure culture study to assess the extent of nitrite accumulation. Given the fact that
476 the experiment lasted for several hours, nitrite accumulation was highly likely. In the presence of
477 $NO_2^-$, $N_2O$ can be produced through $NO_2^-$ reduction, namely the nitrifier denitrification pathway.

478 Thus the SP signature value for the $NH_2OH$ oxidation pathway obtained from the pure culture
479 study can possibly be affected by the nitrifier denitrification pathway. In the mixed culture study,
480 the possible contribution of the nitrifier denitrification pathway was considered. It was assumed
481 that the $N_2O$ emitted at the beginning of the experiments was produced via the $NH_2OH$ oxidation
482 pathway thus generating the $NH_2OH$ oxidation pathway SP signature. However the 'beginning' in
483 this study was not well defined, causing uncertainties in the extent of nitrifier denitrification. The
484 $N_2O$ emitted at 45 min was characterized via SP analysis, and solely attributed to the $NH_2OH$
485 oxidation pathway in experiments where the $NH_2OH$ substrate concentration was 10 mgN/L.
486 However, monitoring showed that 0.4 mgN/L $NO_2^-$ had accumulated at 45 min (Wunderlin et al.
487 2012). The intracellular $NO_2^-$ accumulation is expected to be even higher. The nitrite accumulation
488 effect can be significant considering the nitrite affinity constant for nitrite reduction in activated
489 sludge reported is just 0.14 mgN/L (Law et al. 2012a, Ni et al. 2014). Nitrite accumulation likely
490 occurs as soon as $NH_2OH$ is oxidised, potentially triggering the nitrifier denitrification pathway,
491 and thus likely influencing the SP signature value.

492 In addition to the SP signature value for the $NH_2OH$ oxidation pathways, the SP signature
493 value for the nitrifier denitrification pathway obtained using the substrate separation method is
494 also uncertain. With this method, pure or mixed cultures were only provided with nitrite and
495 ambient air (Sutka et al. 2006, Sutka et al. 2004, Wunderlin et al. 2013a). All $N_2O$ emitted during
496 experiments is assumed to be produced from the nitrifier denitrification pathway since there was
497 no other substrate provided for other pathways. However, it is believed that without $NH_2OH$ as an
498 electron donor, nitrite theoretically cannot be reduced to $N_2O$ by AOB (Hooper 1968, Ritchie and
499 Nicholas 1972). The $N_2O$ emitted during the experiment can possibly be produced via two
500 scenarios. Firstly, $N_2O$ might be produced from both the $NH_2OH$ oxidation pathway and nitrifier

denitrification pathway. Prior to the experiments, the pure culture was incubated with $NH_2OH$ (Sutka et al. 2006). Thus $NH_2OH$ may persist during the batch experiments and act as the electron donor, leading to $N_2O$ emission and contamination of the signature value. Secondly, the $N_2O$ emitted may be produced chemically from the added nitrite. In either scenario, the SP value obtained may not be the reliable signature value for nitrifier denitrification.

To date, enzyme studies potentially provide the only 'uncontaminated' SP values of $N_2O$ production pathways. Yamazaki et al (2014) measured the respective SP values of $N_2O$ produced from $NH_2OH$ oxidation with purified HAO from AOB (*Nitrosomonas europaea* and *Nitrosococcus oceani*), and NO reduction with purified NOR from heterotrophic denitrifier (*Paracoccus denitrificans*). SP values of 36.3 ± 3.3‰ were obtained for the $NH_2OH$ oxidation pathway, higher than previous directly measured signature values, suggesting potential contamination by nitrite reduction in past studies measuring the $NH_2OH$ oxidation pathway SP signature value. The SP value (−5.1±1.8 ‰) obtained from the NO reduction experiment with purified NOR was close to that from the previous $NO_3^-$ reduction experiment (Toyoda et al. 2005). The agreement of SP values suggested that the SP value in the heterotrophic denitrification pathway is controlled by the NO reduction with NOR rather than other steps. Therefore, the SP value obtained by the purified NOR experiment is likely the SP signature value for the heterotrophic denitrification pathway (Yamazaki et al. 2014). The SP value obtained from the purified NOR experiment is similar to the SP signature values measured for the AOB nitrifier denitrification pathway and can potentially be its SP signature value. The *norB* gene of AOB is proven in close orthologous relationship with that of denitrifiers (Casciotti and Ward 2005, Garbeva et al. 2007). But in order to get a reliable SP signature value, experiments with purified NorB from AOB is still required. The SP values obtained from HAO and NOR enzyme studies are

theoretically representative of the corresponding pathways, however, the concern is whether these values can really be practically applied in the complex environment of a WWTS.

Besides direct measurement, other methods have also been used to determine the SP signature value. However, the validity of these methods are still to be established. A pure culture study (*Nitrosomonas marina* C-113a) by Frame and Casciotti (2010) applied $\delta^{18}O\text{-}N_2O$ and SP measurements to estimate the signature values of the $NH_2OH$ oxidation pathway and the nitrifier denitrification pathway. O atoms in the $N_2O$ via the $NH_2OH$ oxidation pathway originate solely from $O_2$ while they are gained from both $O_2$ and $H_2O$ via the nitrifier denitrification pathway. The $\delta^{18}O\text{-}N_2O$ from the nitrifier denitrification pathway is correlated to the changes of $\delta^{18}O\text{-}H_2O$ (Wrage et al. 2005). Therefore, by setting up parallel experiments in $^{18}O$ labelled and unlabelled $^{18}O$, the contribution of the nitrifier denitrification pathway to the $N_2O$ emission was obtained. Thus the mixed SP value of $N_2O$ can be separated by the mixing model to determine the SP signature values of the two sources. However, the reliability of the $^{18}O$ labelling method is in doubt because of the oxygen exchange issue (will be further discussed in Section 5).

### 4.4 Site-preference based quantification method

Site-preference based quantification of $N_2O$ production pathways, like other isotope methods, relies on the mixing model (Frame and Casciotti 2010).

The SP quantification analysis can only apportion the relative contributions of two sources to the products. In WWTS, the SP method has mostly been used in conditions where heterotrophic denitrification is minimal, e.g., in aerated conventional activated sludge systems (Tumendelger et al. 2014, Wunderlin et al. 2013a), and partial nitrification reactors without COD (Harris et al. 2015, Rathnayake et al. 2013). When $N_2O$ produced from two pathways with distinct SP signature values is combined, the relative contribution to the total $N_2O$ emitted can be calculated via the mixture's

SP value. For example, when heterotrophic denitrification is minimal, the relative contribution of the nitrifier denitrification pathway and the $NH_2OH$ oxidation pathway to the total $N_2O$ production can be obtained from Equation 15 by adopting the isotope mixing model (Frame and Casciotti 2010).

$$F_{ND} = (1 - F_{NN}) = \left(\frac{SP_{measure} - SP_{NN}}{SP_{ND} - SP_{NN)}}\right) * 100 \quad (15)$$

where $F_{ND}$ is the relative contribution to total $N_2O$ from the nitrifier-denitrification pathway, $F_{NN}$ is the relative contribution to total $N_2O$ from the $NH_2OH$ oxidation pathway, $SP_{measure}$ is the measured SP value for the $N_2O$ emitted, $SP_{NN}$ is the SP signature value for the $NH_2OH$ oxidation pathway and $SP_{ND}$ is the SP signature value for the nitrifier denitrification pathway. The SP signature values for the $N_2O$ production pathways are the foundation of this quantitative approach.

The $N_2O$ reduction process needs to be corrected in SP-based pathway quantification, since SP values can be shifted by $N_2O$ reduction. $N_2O$ reduction has been proven to increase the SP value of emitted $N_2O$ resulting in an overestimate of the proportion of $N_2O$ emitted from the $NH_2OH$ oxidation pathway (Ostrom et al. 2007). The increase of SP value is due to the preferable $^{14}N$-O bond breakage than the $^{15}N$-O bond. Thus the $^{15}N$ atoms in remaining $N_2O$ molecules are more enriched in the central position (Toyoda et al. 2015). Similar to nitrogen or oxygen isotope enrichment factors discussed in Section 3.4, the variation of SP value for $N_2O$ reduction is described by an enrichment factor, ε(SP), by the simplified Rayleigh equation: $SP = SP_0 + \varepsilon(SP) \ln \frac{C}{C_0}$. SP and $SP_0$ stand for the SP value of $N_2O$ during and before $N_2O$ reduction; C and $C_0$ stand for the concentration of $N_2O$ during and before $N_2O$ reduction, respectively. In the absence of $N_2O$ production, the ratios between enrichment factors (ε) of SP, $\delta^{15}N^{bulk}$ and $\delta^{18}O$ have been observed to be almost constant in pure culture or soil experiments during the $N_2O$ reduction (Koba et al. 2009, Mohn et al. 2012, Ostrom et al. 2007, Yamagishi et al. 2007). The $\varepsilon(^{18}O)/\varepsilon(^{15}N^{bulk})$ and

570 $\varepsilon(^{15}N^{bulk})/\varepsilon(SP)$ ratios for WWTS have been characterized recently to be 2.2 and 0.9, respectively
571 (Tumendelger et al. 2016). By utilizing the covariation of $^{15}N^{bulk}$ and SP values, the shifted SP
572 value by $N_2O$ reduction can be corrected in SP-based pathway quantification (Toyoda et al. 2011b,
573 Yamagishi et al. 2007). The end $\delta^{15}N^{bulk}$ values of the two $N_2O$ production pathways can be
574 obtained by applying the enrichment factor of each pathway to the $\delta^{15}N^{bulk}$ values of source
575 substrates. Then the end $\delta^{15}N^{bulk}$ values and SP signature values of the two $N_2O$ production
576 pathways are used to define a source-mixing equation. Theoretically, if the $N_2O$ reduction is absent
577 or corrected, the line defined by the source-mixing equation passes the SP and $\delta^{15}N^{bulk}$ values of
578 $N_2O$ emitted. Thus the deviated $N_2O$ data by $N_2O$ reduction can be corrected by applying the linear
579 relationship of SP and $\delta^{15}N^{bulk}$ $[\varepsilon(^{15}N^{bulk})/\varepsilon(SP)]$ to intersect with the source-mixing line. With the
580 SP value of the intersection point known, the contribution of the two pathways can be quantified
581 with Equation 15. More details of this correction method can be found in the study of Toyoda et
582 al. (2011b). However, there are still uncertainties with the correction method. As discussed in
583 Section 3.6, the enrichment factors for $N_2O$ production pathways, unlike the SP signature values,
584 are not well-constraint and varies significantly between experiments. The reliability of this method
585 is thus yet to be fully verified and the result is regarded as inaccurate. More research is needed for
586 the correction of $N_2O$ reduction process.

587 **4.5 SP-based quantification of $N_2O$ production pathways in WWTS**

588 Toyoda et al (2011a) introduced the SP method to WWTS, and the method has since found
589 applications to apportion the production of $N_2O$.

590 Although standard SP values for each $N_2O$ production pathway had been determined in
591 pure culture studies (Frame and Casciotti 2010, Sutka et al. 2006, Sutka et al. 2004), there was no

592 link between the SP pure culture studies and the application in WWTS. Thus it is uncertain if the
593 fractionations which occurred in pure culture studies are similar to those in WWTS.

594 In contrast, enrichment factors (Section 3.3) were developed for several nitrogen
595 transformation pathways in wastewater treatment processes, and were found to be similar to those
596 obtained in pure culture studies (Toyoda et al. 2011a). This similarity in enrichment factors may
597 suggest that the $\delta^{15}N$ fractionation behaviour in pure culture studies resembles that in WWTS.
598 Thus the SP value generated by fractionations in pure culture studies should be applicable to
599 WWTS (Toyoda et al. 2011a). Furthermore, a later study by Wunderlin et al (2013a) of mixed
600 culture in WWTS obtaining similar SP signature values to those from pure culture studies (Sutka
601 et al. 2006) suggested the applicability of the SP method in WWTS.

602 Although the SP method has been widely used in WWTS to investigate the origin of $N_2O$,
603 currently there is no universally recognized and characterized SP value for each $N_2O$ production
604 pathway in WWTS. A range of different SP signature values were used in studies as summarized
605 in Table 4.

606 Significant variations in interpreting the results can be caused by using different SP
607 signature values. As illustrated in Figure 4, the estimated the $NH_2OH$ oxidation pathway
608 contributions vary significantly, when the measured SP values change from 0 to 28‰.

609 Despite some inconsistency in choosing SP signature values, some interesting discoveries
610 have been made with applications of the SP method to WWTS. The SP method has been used to
611 study the relative contributions of $N_2O$ production pathways in two types of wastewater treatment
612 systems, namely, conventional activated sludge (CAS) systems and partial nitrification (PN)
613 systems. The CAS system employs full nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$), whereas the partial
614 nitrification system (e.g., PN-anammox and SHARON process) results in $NO_2^-$ as the end-product

615 of nitrification ($NH_4^+ \rightarrow NO_2^-$). The relative contributions of $N_2O$ pathways revealed by SP studies
616 lead to a better apprehension of $N_2O$ pathway dynamics in WWTS.

617 In CAS systems, full-scale studies have shown that in the aeration tank the $NH_2OH$
618 oxidation pathway can contribute up to 90% of total $N_2O$ produced when DO is around 2.5 mg/L,
619 and that nitrifier denitrification contributes similarly where DO is lower at around 1.5 mg/L
620 (Toyoda et al. 2011a, Tumendelger et al. 2014). Where DO concentration is even lower (lower
621 than 1.5 mg/L), the nitrifier denitrification pathway is the major contributor of $N_2O$ emissions
622 (Toyoda et al. 2011a, Tumendelger et al. 2014). This trend has been found to be consistent in
623 laboratory scale studies which suggested increased DO concentration in activated sludge reactors
624 decreased the relative contribution from the nitrifier denitrification pathway, and increased the
625 contribution of the $NH_2OH$ oxidation accordingly (Peng et al. 2014). However, in contrast,
626 laboratory studies showed that the nitrifier denitrification pathway was still the major contributor
627 accounting for approximately 70% of total $N_2O$ emitted even when DO is up to 3 mg/L (Peng et
628 al. 2014, Wunderlin et al. 2013a). In another study, results showed variation of DO between 0.5
629 mg/L and 3.0 mg/L did not alter the $N_2O$ production pathway in WWTS (Tumendelger et al.
630 2016). In fact, all laboratory studies have shown that in $NH_4^-$ oxidation experiments, the nitrifier
631 denitrification pathway is the major contributor to $N_2O$ emission (Azzaya et al. 2014, Tumendelger
632 et al. 2014, Tumendelger et al. 2016, Wunderlin et al. 2013a).

633 In PN systems without COD in the feed, heterotrophic denitrification is assumed to be
634 negligible, and therefore only the $NH_2OH$ oxidation pathway and the nitrifier denitrification
635 pathway contribute to $N_2O$ emission (Harris et al. 2015). In such systems, the $NH_2OH$ oxidation
636 pathway can contribute up to 65% of total $N_2O$ emitted in the initial phase of one cycle, and in the
637 latter phase, the $NH_2OH$ oxidation and the nitrifier denitrification pathways contribute equally

(Rathnayake et al. 2013). Interestingly, in a PN-anammox system, an unexpected high SP value of 45.9‰ was observed (Harris et al. 2015). The value, which is significantly higher than any known $N_2O$ pathway SP values, suggested the possible existence of an unidentified $N_2O$ production pathway in PN-anammox system. In PN systems with COD in the feed, the SP analysis method can only be used to distinguish between $NH_2OH$ oxidation and $NO_2^-$ reduction involving both nitrifier denitrification and heterotrophic denitrification (Ali et al. 2016, Ishii et al. 2014). This is because the SP signature values reported for the nitrifier denitrification pathway and the heterotrophic denitrification pathway are overlapped, making it impossible to distinguish between these two. The $NH_2OH$ oxidation and nitrifier denitrification pathways have both been shown to be important and contribute comparably to total $N_2O$ emissions in PN systems, whereas heterotrophic denitrification might be in some cases the major contributor (Ali et al. 2016, Ishii et al. 2014).

### 4.6 Strengths and limitations of the SP method for application to WWTS

SP analysis is a promising method for quantifying the source of emitted $N_2O$ in WWTS because of the following capabilities:

- SP analysis enables the quantification of the relative contributions of $N_2O$ production pathways,
- SP values are independent of substrate isotopic compositions and bulk $\delta^{15}N$ fractionations thus quantitatively reflecting the origin of $N_2O$,
- Unlike the inhibition approach or labelled isotope analysis which introduces inhibitors or artificially enriched $^{15}N/^{18}O$, no foreign influences are introduced as SP analysis is based on natural $^{15}N$ abundance, and

- SP analysis provides direct evidence investigating $N_2O$ production pathways. Thus SP analysis can be a powerful tool in combination with other indirect methods such as enzyme mRNA transcription analysis (Section 6.1).

Despite the capabilities of the SP method, application of the SP method in WWTS is still challenging. While the SP method can quantify the contributions of $N_2O$ production pathways, the accuracy of the quantification results is not guaranteed. The inaccuracy of SP method quantification can be caused by measurement errors, controversial SP signature values, the simultaneous occurrence of $N_2O$ reduction and the presence of unidentified $N_2O$ production pathways.

SP measurement errors and poorly-defined SP signature values for $N_2O$ production pathways contribute most to the inaccuracy of SP method quantification. For currently available $N_2O$ isotopomer measurement techniques (IRMS and QCLAS), there is an unresolved discrepancy between the measurement results from different laboratories which is likely to result in large variations in $N_2O$ pathway quantifications. The discrepancy is mostly due to the lack of measurement standards including a standard calibration strategy, a standard quantification algorithm and most importantly, standard $N_2O$ isotopomer reference materials. As suggested by a recent inter-laboratory study by Mohn et al. (2014), the calibration strategy and quantification algorithm vary considerably among laboratories. The reference material is essential to improve the analytical techniques because it enables the validation of the calibration strategy and quantification algorithm. Apart from the lack of standards, some other effects, e.g., the sample pre-treatment and the spectrometer configuration can also affect the accuracy of SP isotopomer measurement (Mohn et al. 2014). The inter-laboratory study involving eleven participating laboratories employing IRMS or QCLAS, aimed at assessing the isotopomer measurement methods showed that the

683 measured SP values of one target gas varied from 15.14‰ to 25.45‰ with a standard variation of
684 4.24% (Mohn et al. 2014). If these SP values were used to determine the $N_2O$ origin using the
685 method adopted by Rathnayake et al (2013), the contribution of the $NH_2OH$ oxidation pathway
686 would be within the broad range of 45.9% to 77.1%, representing a large uncertainty caused by
687 measurement errors. The measurement inaccuracy can be compounded by uncertainties related to
688 SP signature values (Section 4.5). To enhance the quantification accuracy of the SP method,
689 standard reference gases with different SP values should be produced and distributed to
690 laboratories for calibration and measurement validation. A universally accepted measurement and
691 calibration protocol would also help to address the issue. Besides, a set of universally accepted SP
692 signature values for each $N_2O$ production pathway in WWTS should be established.

693 $N_2O$ reduction can shift the SP value thus affecting the accuracy of the SP method (Section
694 4.4). Although it is theoretically possible to correct the effects of $N_2O$ reduction, the correction
695 method is unsound. Therefore the accuracy of SP-based pathway quantification will be affected
696 by potential $N_2O$ reduction in WWTS.

697 Furthermore, $N_2O$ production pathways other than the three main pathways, e.g.
698 dissimilatory nitrate reduction to ammonium and N-nitrosation hybrid $N_2O$ pathway, have not been
699 considered in the quantification so far. For example, Harris et al (2015) reported unexpectedly high
700 SP values of up to 45.9‰ suggesting the influence of alternative unidentified $N_2O$ production
701 pathways. These unidentified pathways can influence the SP values thus deteriorating the
702 quantification accuracy.

703 In addition to the accuracy issues, there may not exist a set of universal SP signature values
704 for $N_2O$ production pathways in WWTS. The existence of universal SP signature values in WWTS
705 is based on the assumption that the $^{15}N$ fractionations occurred in WWTS are similar to that in

706 pure cultures. This assumption is based on indirect evidence that the $^{15}N$ enrichment factors in
707 WWTS resemble those in pure culture studies (Toyoda et al. 2011a). However, as discussed, the
708 SP value is dependent on the site-preference fractionation which, in turn, may not necessarily
709 correlate to the bulk $^{15}N$ fractionation. Thus, the SP fractionations in mixed culture activated
710 sludge of WWTS do not necessarily reflect that of pure cultures, and assuming universal SP
711 signature values for each pathway may not be valid. Furthermore, only one study (Wunderlin et
712 al. 2013a) has been done so far to determine the SP signature values for $N_2O$ production pathways
713 in WWTS. Given the complex composition of activated sludge and its variation between plants, it
714 is possible that there is not a universal SP signature value for each $N_2O$ production pathway. This
715 foundation needs to be validated carefully by performing more experiments directly with activated
716 sludge to determine more SP signature values of activated sludge from a variety of WWTS.
717 Finally, in WWTS, the SP method can only distinguish the relative contributions of the
718 $NH_2OH$ oxidation pathway and the nitrifier denitrification pathway when the heterotrophic
719 denitrification pathway is negligible. The SP method does not allow simultaneous separation of
720 the three main microbial production pathways because the mass balance equations based on SPs
721 would not give a unique solution for three unknowns. Furthermore, the nitrifier denitrification
722 pathway and the heterotrophic denitrification pathways cannot be separated because the two
723 processes have very similar SP signature values (Sutka et al. 2006, Toyoda et al. 2011a, Toyoda
724 et al. 2011b). Similarly, if the abiotic reactions are also considered, the relative contributions by
725 the abiotic reactions (include the N-nitrosation hybrid $N_2O$ production pathway) and the $NH_2OH$
726 oxidation pathway are not distinguishable with the SP method because of the overlapping SP
727 signature values. The differentiation of the two pathways with similar SP values may be achieved
728 by using artificially enriched $^{15}N$ or $^{18}O$ substrates with tracer method (*vide infra*).

## 5. $N_2O$ production pathways quantification based on tracer method

The natural abundance and site preference approaches are based on naturally occurring substrate. In comparison, the tracer method uses artificially enriched isotopic substrates as labels to trace the $N_2O$ production pathways. The natural abundance and SP methods rely on isotopic fractionation and isotope mixing. For the tracer method, isotopic fractionation is negligible because it only slightly changes the $^{15}N$ abundance (Fry 2006). Instead, the isotope composition in the product is regarded as solely dependent on the isotope mixing. Thus enriched isotopic substrate can be used to trace the $N_2O$ production pathway, such as in the definitive study by Richie and Nicholas (1972) using $^{15}N$ enriched $NO_2^-$ to trace the nitrifier denitrification pathway where they proved the existence of the nitrifier denitrification pathway. Apart from tracing, this method can also be used to quantify $N_2O$ production pathways (Zhao et al. 2014).

With the $^{15}N$-tracer method,$^{15}N$ enriched $NH_4^+$ or $NO_3^-$ substrates were implemented to determine the relative contribution of nitrification and denitrification; however, this approach is not much used to date due to its drawbacks. It is assumed that the N atom in the $N_2O$ emitted during nitrification arises from $NH_4^+$, while in denitrification the N atom comes from $NO_3^-$. Since the artificial isotopic compositions of $^{15}N$ in $NH_4^+$ and $NO_3^-$ can be distinguished, the $N_2O$ production contributions from nitrification and denitrification, respectively, can be calculated using the mixing model (Itokawa et al. 2001). However, the nitrifier denitrification pathway is not considered in this approach since it is not possible to distinguish it from the heterotrophic denitrification pathway using $^{15}N$-$NO_3^-$. During heterotrophic denitrification, the N atom in the emitted $N_2O$ can possibly be from substrates, nitrite or nitrate, produced during nitrification. Thus the N atoms in $N_2O$ produced via the heterotrophic denitrification pathway could potentially originate from $NH_4^+$ via the nitrification pathway. Consequently, there are flaws in the

752 interpretation of results and it is inherently inconclusive to separate the three main microbial $N_2O$
753 production pathways with this method.

754 While realizing its limitations, the $^{15}N$-tracer method has its strength to quantify the
755 contributions of the recently recognized N-nitrosation hybrid $N_2O$ production pathway, the
756 $NH_2OH$ oxidation pathway and the nitrifier denitrification pathway when heterotrophic
757 denitrification is negligible (Terada et al. 2017). In this method, $^{15}N$ labelled $NH_2OH$ and $NO_2^-$,
758 the direct substrates for the three pathways of interest, were used as tracers. The N atoms in the
759 $N_2O$ emitted from $NH_2OH$ oxidation and nitrifier denitrification arise entirely from $NH_2OH$ and
760 $NO_2^-$, respectively. The N atoms in the $N_2O$ produced from the N-nitrosation hybrid $N_2O$
761 production pathway originate, however, equally from $NH_2OH$ and $NO_2$-. Through the three
762 pathways of interest, three $N_2O$ isotopocules with m/z at 44, 45 and 46 are produced and their
763 respective ratios obtained. Along with the known and distinct $^{15}N$ isotopic compositions in the
764 substrates $NH_2OH$ and $NO_2^-$, the contributions of each pathway can be quantified. To minimize
765 the limitation of the N atom in $NO_2^-$ from $NH_2OH$, the isotopic composition of $^{15}N$ in the substrates
766 are measured with time. With the abundance of $N_2O$ isotopocules also measured with time, the
767 contributions of each pathway are finally calculated with a trapezoidal method. This tracer-based
768 quantification contributes to the comprehension of the N-nitrosation hybrid $N_2O$ production
769 pathway in WWTS.

770 Combining $^{15}N$ with $^{18}O$ tracer makes it theoretically possible to differentiate the three
771 main microbial $N_2O$ production pathways (Kool et al. 2011, Wrage et al. 2005). The O atom in a
772 $N_2O$ molecule can originate from $O_2$, $H_2O$ or $NO_3^-$ molecules. When $NH_4^+$ is oxidized by $O_2$ to
773 $NH_2OH$, the O atom in $NH_2OH$ is solely from $O_2$. When $NH_2OH$ is further oxidized to $NO_2^-$, the
774 second O atom is supplied by $H_2O$. Therefore, the O atoms in $NO_2^-$ molecules are 50% from $O_2$

775 and 50% from $H_2O$. When $NO_2^-$ is then oxidized to $NO_3^-$ another O atom is gained from $H_2O$ so
776 that 67% of the O atoms in $NO_3^-$ are from $H_2O$ while 33% from $O_2$. $N_2O$ produced during nitrifier
777 denitrification, $NH_2OH$ oxidation and heterotrophic denitrification is assumed to be originally
778 converted from $NO_2^-$, $NH_2OH$ and $NO_3^-$, respectively. The different percentage of O atom sources
779 in precursors coupled with $^{15}N$ tracer enables the differentiation of the $N_2O$ production pathways
780 through the mixing model (Phillips and Koch 2002).

781 However, the $^{18}O$- and $^{15}N$-combination method also has limitations, and to date has not
782 been applied in WWTS. Firstly, the origin of $N_2O$ produced from heterotrophic denitrification can
783 be $NO_2^-$ which is also the origin of $N_2O$ produced through the nitrifier denitrification pathway.
784 Thus this approach can overestimate the contribution of the nitrifier denitrification pathway to the
785 total $N_2O$ emission while underestimate that of the heterotrophic denitrification pathway.
786 Secondly, a question is often raised regarding the possible effect of oxygen exchange between $H_2O$
787 and intermediates of $N_2O$. Oxygen exchange has an important role in determining oxygen isotope
788 (Kool et al. 2009) , thus causing bias in estimation using the $^{18}O$ tracer method. In particular, it has
789 been reported that for many abundant nitrifying- and denitrifying bacteria in nature, the exchange
790 rate between $NO_2^-$ and $H_2O$ can reach up to 100% (Kool et al. 2007). Recently, a method named
791 enrichment ratio retention (ERR) was proposed using $NO_3^-$ enriched in both $^{18}O$ and $^{15}N$ to
792 quantify the oxygen exchange during denitrification (Kool et al. 2011). The principal is that in
793 denitrification, if no oxygen is exchanged with non-enriched $H_2O$, the ratio of $^{18}O$: $^{15}N$ in the
794 resulting $N_2O$ molecule should be the same as in the original $NO_3^-$. Thus by assessing the change
795 in $^{18}O$:$^{15}N$ ratio, the percentage of O that has been exchanged can be quantified. However, there is
796 still no applicable approach to assess the oxygen exchange during nitrification, where oxygen

797 exchange also happens. Thus using the $^{18}O$ tracer method for $N_2O$ source determination in
798 nitrification can be unreliable and should be used with caution.

799 **6. Opportunities for isotope technology in combination with other methods**

800 Some limitations of isotope technology are inherent, and may not be resolved by combining
801 isotope approaches. Indeed the SP method itself cannot quantify the relative contributions from
802 more than two $N_2O$ production pathways, the $^{15}N$ tracer method itself cannot reliably quantify the
803 main $N_2O$ production pathways, and isotope technology cannot predict the $N_2O$ emission and
804 pathway origins based on varying conditions. The opportunity to enhance quantification of $N_2O$
805 production pathways using isotope technologies lies in combining the isotope methods with other
806 complementary approaches. For instance, the SP method combined with mRNA based
807 transcription analysis enabled the differentiation of the three main pathways (Ishii et al. 2014), the
808 $^{15}N$ tracer method combined with inhibition method are able to quantify the nitrifier denitrification
809 pathway and the heterotrophic denitrification pathway (Zhao et al. 2014), and SP analysis
810 combined with mathematical $N_2O$ models can make the $N_2O$ production pathway prediction in
811 WWTS more reliable (Peng et al. 2014). While these methods are yet to become mature, the
812 combinatory approach has already yielded promising results.

813 **6.1 SP analysis combined with mRNA based transcription analysis**

814 Investigation of microbial gene expression helps to identify the $N_2O$ production pathways
815 by revealing how the microbial activities are regulated. $N_2O$ production pathways are a function
816 of their related enzymes, which are encoded and regulated by mRNA transcribed from
817 corresponding bacterial DNA. Although quantification of enzymes can provide the most direct
818 evidence on production pathways, it is still challenging to quantify nitrifying and denitrifying
819 enzymes (Philippot and Hallin 2005). Studying the expression of functional genes via mRNA

measurement is a viable alternative for studying the regulation of pathways. Compared with DNA-based Denaturing Gradient Gel Electrophoretic analysis (DGGE), which is effective for analyzing the composition of species like the nitrifying and denitrifying bacterial populations (Hu et al. 2011, Tam et al. 2005), mRNA based transcription analysis can estimate microbial activities. This is because, firstly mRNA is transcribed from functional DNA, and secondly, the short half-life of mRNA in prokaryotes (Philippot and Hallin 2005) can reflect the regulation processes. Of the techniques used for quantifying mRNA, quantitative reverse transcription polymerase chain reaction (RT-qPCR) has often been utilized to study the $N_2O$ production pathways (Song et al. 2014, Yu et al. 2010). When RT-qPCR is used to target the mRNA encoding a particular enzyme, the activity of the enzyme in the pathway can be quantified. For example, the RT-qPCR method has been used to study pathway changes during transient anoxia in *N. europaea* (Yu et al. 2010). The expression of four functional genes encoding: AmoA for $NH_4^+$ oxidation, HAO for $NH_2OH$ oxidation, NirK for $NO_2^-$ reduction and NorB for nitric oxide reduction was measured. The changes in gene expression indicate bacterial regulation strategies in each pathway under anoxia conditions. For example, the increase in *nirK* expression during transient anoxia suggests upregulation of the nitrifier denitrification pathway.

Ishii et al (2014) made progress in combining mRNA based transcription analysis with SP analysis to determine the relative contribution of three main $N_2O$ production pathways. The mRNA based transcription analysis distinguished the contributions of AOB and heterotrophic denitrifiers to $N_2O$ emission in WWTS. The amount of *Nitrosomonas*-related *cnorB* transcript was measured as a proxy for the amount of *Nitrosomonas*-related NorB expressed, representing the nitric oxide reduction activity of AOB, and thus the $N_2O$ emitted by AOB. Because *norB* in AOB and *norB* in heterotrophic denitrifiers can be differentiated, the NO reduction activity by heterotrophic

843 denitrifiers can also be measured. Thus, using mRNA based transcription combined with SP
844 analysis, which differentiates the contribution from $NH_2OH$ oxidation and denitrification by AOB
845 and heterotrophic denitrifiers, the contributions of the three pathways to $N_2O$ production in WWTS
846 can be assessed.

847 However, some studies have shown that functional gene transcription does not necessarily
848 reflect the corresponding enzyme activity level. For instance, a study on denitrification (Henderson
849 et al. 2010) demonstrated no significant relationship between $N_2O$ emission and denitrifying
850 functional gene transcription levels. Specifically, the crucial primer selection process limited by
851 the metabolism knowledge can influent the reliability of the interpretation. Yu and Chandran
852 (2010) studied transcription level changes of four *Nitrosomonas europaea* functional genes (*amoA*,
853 *hao*, *nirK* and *norB*) under different DO and nitrite concentrations. The profile of *nirK* was not
854 found to parallel the corresponding nitrite reduction level. The differences between gene
855 transcription and activity levels may partly be attributed to the imperfect primer selection as an
856 unidentified nitrite reductase alternative to NirK was found recently (Kozlowski et al. 2014). Thus,
857 while mRNA transcription can be used as an indicator, other techniques such as the intermediate
858 chemical monitoring and SP analysis are needed to confirm results.

859 **6.2 $^{15}N$ tracer method combined with metabolic inhibitors**

860 Inhibition methods have been used in WWTS to understand $N_2O$ production pathways
861 through utilizing metabolic inhibitors that act on specific processes in nitrification or
862 denitrification (Hu et al. 2011, Tallec et al. 2006). In WWTS, inhibitors that are widely used for
863 investigating $N_2O$ production pathways include acetylene ($C_2H_2$), allylthiourea (ATU), $NaClO_3$
864 and dicyandiamide (DCD). ATU and $NaClO_3$ are usually used in combination to specifically
865 inhibit nitrification to distinguish it from denitrification in WWTS (Tallec et al. 2008). ATU can

inhibit the first step of nitrification, i.e., the oxidation of $NH_4^-$ to $NO_2^-$, by inhibiting AMO, while $NaClO_3$ can inhibit the second step of nitrification, i.e., the oxidation of $NO_2^-$ to $NO_3^-$ by inhibiting the nitrite oxidoreductase (Rusmana and Nedwell 2004). Zhao et al (2014) illustrated how the $^{15}N$ tracer method combined with experiments with- or without inhibitors led to quantification of the nitrifier denitrification and heterotrophic denitrification pathways. Because DCD can inhibit both AMO and NirK (Shapleigh and Payne 1985), DCD can also be used in WWTS with ATU to study the role of NirK in nitrification (Kim et al. 2010). Acetylene has been used to inhibit $N_2O$ reductase in SP analysis as $N_2O$ reduction may bias the SP contribution studies (Toyoda et al. 2005).

However, inhibition methods do have limitations. It is possible for inhibitors to affect other pathways in addition to the targeted pathways. For example, under aerobic conditions, $NaClO_3$ is utilized in WWTS to inhibit nitrite oxidoreductase thus inhibiting the second step of nitrification (Zhao et al. 2014). However, $NaClO_3$ also inhibits nitrate reductase (NAR) in denitrifiers (Hall 1984). NAR and NAP are two biochemically distinct nitrate reductase. True denitrifiers have NAP with or without NAR for nitrate reduction (Richardson et al. 2001). Thus the inhibitor can also affect the heterotrophic denitrification pathway leading to biased estimation of nitrification. Besides, inhibition methods have been reported in some studies ineffectively inhibiting $N_2O$ production (Tilsner et al. 2003, Wrage et al. 2004). Furthermore, inhibitors may react with intermediates of the $N_2O$ production pathways thus affecting the reliability of the method. For example, chlorite, which selectively inhibit formation of $N_2O$ from nitrite, can chemically oxidize nitrite to nitrate (Rusmana and Nedwell 2004).

### 6.3 Isotope technologies combined with mathematical modeling

Mathematical modeling is a useful tool for understanding $N_2O$ production in WWTS. It can predict and simulate $N_2O$ production, and the contribution from various pathways (Ni and

889 Yuan 2015). The mechanistically based mathematical modeling of $N_2O$ production relies on the
890 knowledge of $N_2O$ production pathways occurring in WWTS. Electron mass balance and/or energy
891 (ATP) balance were used to link nitrogen transformation reactions to pathways. Pathway
892 knowledge is the essential difference between $N_2O$ production models. Some $N_2O$ production
893 models for AOB are based on a single pathway: either the nitrifier denitrification pathway or the
894 $NH_2OH$ oxidation pathway (Law et al. 2012a, Ni et al. 2013); while others incorporate both
895 pathways (Ni et al. 2014, Peng et al. 2015a, Peng et al. 2014, Pocquet et al. 2016). The two-
896 pathway AOB $N_2O$ production models are applicable in a wider range of conditions in comparison
897 to the single pathway models (Ni and Yuan 2015). However, the validation of the two-pathway
898 models is challenging due to the lack of pathway contributions data. This can affect the reliability
899 of the predicted result. The first two-pathway AOB $N_2O$ production model proposed by Ni et al.
900 (2014) was only validated by the total $N_2O$ emission data. While the model could successfully
901 describe the total $N_2O$ emission, the prediction of the two pathway contributions is uncertain in
902 the absence of more direct evidence.

903 Isotope analysis is likely the best experimental method for providing direct evidence to
904 validate model predictions. Peng et al (2014) enhanced the reliability of the pathway-contribution
905 prediction by combining the two-pathway AOB $N_2O$ model with SP analysis. The two-pathway
906 $N_2O$ model of Ni et al. (2014) was chosen in this study to interpret the experiment results of DO
907 effects on $N_2O$ production pathways. The model was calibrated using the two-step calibration
908 procedure by fitting simulation results to the $N_2O$ emission experimental data under different DO
909 concentrations. At the same time, the relative contributions of two AOB pathways were obtained
910 with the SP method. Finally, the model predicting relative contributions of the two pathways for
911 $N_2O$ production were verified with the experimental data. The verified two-pathway model can be

912 used to more reliably predict the contributions of the two $N_2O$ production pathways of AOB under
913 a broad range of conditions, and forms a powerful tool for future $N_2O$ studies.

914 **7. Conclusions and future perspectives**

915 Here, the isotope technologies available for studying the $N_2O$ production pathways in
916 WWTS have been critically reviewed to illustrate their capabilities and limitations. The purpose
917 of this review was to identify the potential challenges with utilizing these technologies, and where
918 possible, to propose improvements to the approaches. The key conclusions are:

919 • Conventional natural stable isotope studies including enrichment factor studies are of
920 limited use for quantifying $N_2O$ production pathways in WWTS because of the interference
921 of non-enzymatic fractionations irrelevant to nitrogen conversion pathway.

922 • Site-preference analysis is currently the most promising technology for quantifying the
923 relative contributions of $N_2O$ production pathways although its accuracy still needs
924 improvement. In particular, the SP measurement accuracy should be improved and the SP
925 signature values for different pathways should be more accurately established.

926 • $^{15}N$ tracer can reliably trace the nitrogen conversion processes. However, since various
927 $N_2O$ production pathways occur simultaneously in WWTS, it is not possible to quantify
928 each of them with $^{15}N$ tracer alone. When $^{15}N$ tracer is combined with $^{18}O$ tracer, it is
929 possible to quantify the relative contribution of the three main microbial $N_2O$ production
930 pathways. Nevertheless, because of oxygen exchange, caution should be exercised when
931 interpreting results from $^{18}O$ tracer studies.

932 Although there are limitations to the application of isotope technologies, their future use
933 will significantly broaden our understanding of the $N_2O$ production pathways in WWTS. In order
934 to achieve this, the following future efforts are necessary:

- Improving SP measurement accuracy through the production of standard reference materials and establishment of universally accepted calibration and measurement protocols. Reference materials are essential for calibration and can be used for validation of measurement approaches. Another inter-laboratory measurement assessment study can then be carried out to examine the calibration and measurement protocols, which should involve international collaboration.
- Developing an approach that can reliably isolate the $N_2O$ production pathway of interest so that the $N_2O$ production pathway SP signature values for WWTS can be determined. The chemical inhibitor can be introduced but needs to be treated with caution because of its potential unexpected effects on the pathway of interest. Some environmental conditions which have inhibitory effects on some $N_2O$ production pathways can also be considered. For example, high DO may be used to inhibit the nitrifier denitrification pathway and heterotrophic denitrification pathway leading to isolation of the $NH_2OH$ oxidation pathway.
- Validating the universality of SP signature values in WWTS. SP signature values for $N_2O$ production pathways need to be determined for different activated sludge from various WWTS.
- Using isotope technologies in combination with other methods (e.g. RT-qPCR, inhibition and modeling) to overcome the limitations and harness the full potential of those methods.

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1343 **List of tables**

1344 **Table 1** Natural isotope values obtained in WWTS.

| | $\delta^{15}N$ (‰) | $\delta^{18}O$ (‰) | Reference |
|---|---|---|---|
| Oxic tank | -19.7 ± 4.9 | 41.1 ± 9.5 | Toyoda et al. (2011a) |
| Anaerobic tank | 2.2 ± 4.9 | 47.6 ± 6.5 | Toyoda et al. (2011a) |
| Anoxic tank | 5.4 ± 2.8 | 35.4 ± 8.2 | Toyoda et al. (2011a) |
| Oxic tank | -24.5 ± 2.2 | 36.3 ± 3.2 | Townsend-Small et al. (2011) |
| Anoxic tank | 0.0 ± 4.0 | 45.0 ± 2.4 | Townsend-Small et al. (2011) |

1346 **Table 2** $\delta^{15}N$ and $\delta^{18}O$ enrichment factors for different $N_2O$ production pathways

| Pathways | Bacteria | $\varepsilon(^{15}N)$(‰) | $\varepsilon(^{18}O)$(‰) | Reference |
|---|---|---|---|---|
| **$NH_2OH$ oxidation pathway** | *Methylococcus capsulatus Bath* | -0.3 to 4.8 | na | Sutka et al. (2003) |
| | *Methylosinus trichosporium* | 5.7 | na | Sutka et al. (2006) |
| | *Nitrosomonas europaea* | 2.0 | na | Sutka et al. (2006) |
| | *Nitrosospira multiformis* | 2.0 | na | Sutka et al. (2006) |
| | WWTP mixed culture | -5.6 | | Wunderlin et al. (2013a) |
| | *Nitrosomonas marina* C-113a | na | -2.9 ± 0.8 | Frame and Casciotti (2010) |
| | HAO extracted from *Nitrosomonas europaea* | -8.1 ± 1.4 | na | Yamazaki et al. (2014) |
| | HAO extracted from *Nitrosococcus oceani* | -10.5 ± 0.6 | na | Yamazaki et al. (2014) |
| **Heterotrophic denitrification pathway** | *Pseudomonas aureofaciens* | na | 40 | Casciotti (2009) |
| | *Paracoccus denitrificans* | -22 to -10 | 4 to 23 | Toyoda et al. (2005) |
| | *Pseudomonas aureofaciens* | -36.7 | na | Sutka et al. (2006) |
| | *Pseudomonas chlororaphis* | -12.7 | na | Sutka et al. (2006) |
| | *Pseudomonas fluorescens* | -37 to -33 | na | Yoshida et al. (2012) |
| | WWTP mixed culture | -5.6 ± 4.0 | 2.2 ± 2.4 | Townsend-Small et al. (2011) |
| | WWTP mixed culture | 12.1 ~ 19.5 | | Wunderlin et al. (2013a) |
| **Nitrifier denitrification pathway** | *Nitrosomonas europaea* | -39.5 to -31.4 | na | Sutka et al. (2003) |
| | *Nitrosomonas marina* C-113a | - 56.9 ± 3.8 | 8.4 ± 1.4 | Frame and Casciotti (2010) |
| | WWTP mixed culture | 24.4 to 29.2 | na | Wunderlin et al. (2013a) |
| **$NH_4^+$ oxidation ($NH_2OH$ oxidation and nitrifier denitrification pathways)** | WWTP Mixed culture | 43.5 to 58.8 | na | Wunderlin et al. (2013a) |
| | WWTP Mixed culture | -30.1 ± 2.2 | -6.5 ± 3.2 | Townsend-Small et al. (2011) |
| | WWTP Mixed culture | -11.2 to -7.9 | -5.3 to -4.1 | Toyoda et al. (2011a) |
| | WWTP Mixed culture | -14.1 | -5 to -4 | Tumendelger et al. (2016) |

1347

1348 **Table 3** SP signature values obtained for different $N_2O$ production pathways. *For $N_2O$ reduction (see Section 4.4), isotopic

1349 enrichment factors for SP values are shown.

| Pathway | Method | Bacteria | SP or ε(SP) (‰)* | Remarks | Reference |
|---|---|---|---|---|---|
| **$NH_2OH$ oxidation pathway** | Pure culture | *Methylococcus capsulatus* | 30.8 ± 5.9 | Concentrated purified bacteria were incubated with $NH_2OH$ (For *Methylococcus capsulatus*, nitrate mineral salts was added). Air in headspace. For AOB experiments nitrite accumulation was not measured. $N_2O$ possibly produced by $NO_2^-$ reduction was not considered. | |
| | | *Nitrosomonas europaea* | 14.9 ± 3.7 | | Sutka et al. (2004) |
| | | *Nitrosomonas europaea* | 33.5 ± 1.2 | | Sutka et al. (2006) |
| | | *Nitrosospira multiformis* | 32.5 ± 0.6 | | |
| | | *Methylosinus trichosporium* | 35.6 ± 1.4 | | |
| | Mixed culture | WWTS Mixed culture | 28.4 ± 1.9 | Activated sludge sampled from an aerobic reactor was incubated with $NH_2OH$ at different DO levels. The SP of $N_2O$ produced at the beginning of the experiments was regarded as the signature value for $NH_2OH$ oxidation. | Wunderlin et al. (2013a) |
| | Pure enzyme | HAO enzyme from *Nitrosomonas europaea* | 36.6 ± 3.3 | HAO extracted was incubated with $NH_2OH$ for 2h at 25℃, $NO_2^-$ was determined afterwards. This approach did not consider the effect of other processes on fractionation. | |
| | | HAO enzyme from *Nitrosococcus oceani* | 36.2 ± 1.7 | | Yamazaki et al. (2014) |
| | Indirect | *Nitrosomonas marina* c-113a | 36.6 ± 2.4 | Signature values estimated from the SP mixing model based on the quantification of the nitrifier denitrification pathway by parallel experiments with and without $^{18}O$ enriched water. However, oxygen exchange may discredit this approach. | Frame and Casciotti (2010) |
| | | Estimate | 33 ± 4 | Estimation based on previous literature reported value. | Toyoda et al. (2011b) |
| **Nitrifier denitrification** | Pure culture | *Nitrosomonas europaea* | -0.8 ± 5.8 | Concentrated purified bacterial suspension was incubated with $NO_2^-$. Headspace was purged with $N_2$. | Sutka et al. (2004) |
| | | *Nitrosospira multiformis* | 0.1 ± 1.7 | $NO_2^-$ was added to the bacterial suspension. Interestingly, no electron donor was present in this experiment even though $N_2O$ was produced suggesting | Sutka et al. (2006) |

| | | | | | |
|---|---|---|---|---|---|
| | | | | possible contamination with residual $NH_2OH$ from the pre-incubation phase. | |
| | Mixed culture | WWTS Mixed culture | -1.7 ± 1.3 | Activated sludge sampled from the aerobic reactor was incubated with $NO_2^-$ at different DO levels. The average SP of $N_2O$ produced during the experiments was regarded as the signature value. | Wunderlin et al. (2013a) |
| | Indirect | *Nitrosomonas marina* c-113a | -10.7 ± 2.9 | Signature values estimated from the SP mixing model based on quantification of the nitrifier denitrification pathway by parallel experiments with and without $^{18}O$ enriched water. However, oxygen exchange may discredit this approach. | Frame and Casciotti (2010) |
| | | Estimate | 0.4 ± 5.5 | Estimation based on values previously reported in literature. | Toyoda et al. (2011b) |
| **Heterotrophic denitrification** | Pure culture | *Pseudomonas fluorescens* | 23.3 ± 4.2 | Concentrated bacterial suspension was incubated with $NO_3^-$ and COD. The liquid and gas phases were purged with $C_2H_2$/He (10% v/v) to ensure anoxic condition and also prevent $N_2O$ reduction. High SP value from *P. fluorescens* might have resulted from non-enzymatic abiotic formation due to low $N_2O$ production rate. | Toyoda et al. (2005) |
| | | *Paracoccus denitrificans* | -5.1 ± 1.8 | | |
| | | *Pseudomonas chlororaphis* | -0.5 ± 1.9 | Concentrated bacterial suspension in citrate minimal medium was incubated with $NO_3^-$. The headspace was purged with $N_2$. Potential for $N_2O$ reduction which could contaminate the signature value. | Sutka et al. (2006) |
| | | *Pseudomonas aureofaciens* | -0.5 ± 0.6 | | |
| | | *Pseudomonas chlororaphis* | -0.6 ± 1.9 | Concentrated bacterial suspension in citrate minimal medium was incubated with $NO_2^-$. The headspace was purged with $N_2$. Potential for $N_2O$ reduction which could contaminate the signature value. | Sutka et al. (2006) |
| | | *Pseudomonas aureofaciens* | -0.5 ± 1.9 | | |
| | Pure enzyme | NOR enzyme from *Paracoccus denitrificans* | -5.9 ± 2.1 | NOR extracted was incubated with NO for 2h at 25℃. This approach did not consider the effect of other processes on fractionation. | Yamazaki et al. (2014) |
| | Mixed culture | WWTS Mixed culture | 25 | Activated sludge sampled from the aerobic reactor was incubated with $NO_3^-$ and COD at zero or low DO. The method was based on the assumption that $N_2O$ reductase was inhibited by low DO and the presence of $NO_2^-$. | Wunderlin et al. (2013a) |

| | | | | | |
|---|---|---|---|---|---|
| | Indirect | Estimate | -2.0 ± 3.4 | Estimation based on value previously reported in literature. | Toyoda et al. (2011b) |
| **$N_2O$ reduction*** | Pure culture | *Pseudomonas stutzeri*<br>*Pseudomonas denitrificans* | -5.0<br>-6.8 | Pure culture was incubated with isotopically characterized $N_2O$. SP enrichment factors were measured. 10% of total $N_2O$ reduction increased SP by 0.9‰. | Ostrom et al. (2007) |
| | Mixed culture | WWTS Mixed culture | -10.0 ± 2.2 | Activated sludge from anoxic tank was kept under anoxic conditions. $N_2O$ standard gas was bubbled in. Experiment was started by adding COD. SP increased from -2.7 to +20.9‰ during the experiment period (30min). | Tumendelger et al. (2016) |
| | Indirect | Estimate | -5.9 ± 0.9 | Estimation based on values previously reported in literature. | Toyoda et al. (2011b) |
| **Abiotic reactions** | Abiotic | Inorganic | 30.1 ± 1.7 | $NO_2^-$ inorganic reduction with $(CH_3)_3NBH_3$ | Toyoda et al. (2005) |
| | | | 29.5 ± 1.1 | $[NH_3OH]Cl$ abiotic oxidation with $MnO_2$ | |
| | | | 30.3 ± 0.2 | $NH_2OH$ added to tap water | Wunderlin et al. (2013a) |
| | | | 33.9 to 35.2 | A series of experiments measuring the SP values of $N_2O$ produced from the interactions of $NH_2OH$ with $NO_2^-$, $NH_2OH$ with $Fe^{3+}$, $NH_2OH$ with $Cu^{2+}$, $NH_2OH$ with $NO_2^-$ and $Fe^{3+}$, $NH_2OH$ with $NO_2^-$, $Fe^{3+}$ and $Cu^{2+}$ were conducted. Relatively constant results are obtained. | Heil et al. (2014) |

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1351 **Table 4** SP signature values used in WWTS studies

| Studies in WWTS | SP signature value used for the $NH_2OH$ oxidation pathway, ‰ | SP signature value used for the nitrifier denitrification pathway, ‰ |
|---|---|---|
| **a**: Rathnayake et al. (2013) | 33 | 0 |
| **b**: Peng et al. (2014); Wunderlin et al. (2013a) | 28.5 | -2 |
| **c**: Toyoda et al. (2011a) | 29 | -14 |
| **d**: Tumendelger et al. (2014); Ali et al. (2016) | 33 | -1 |

1352

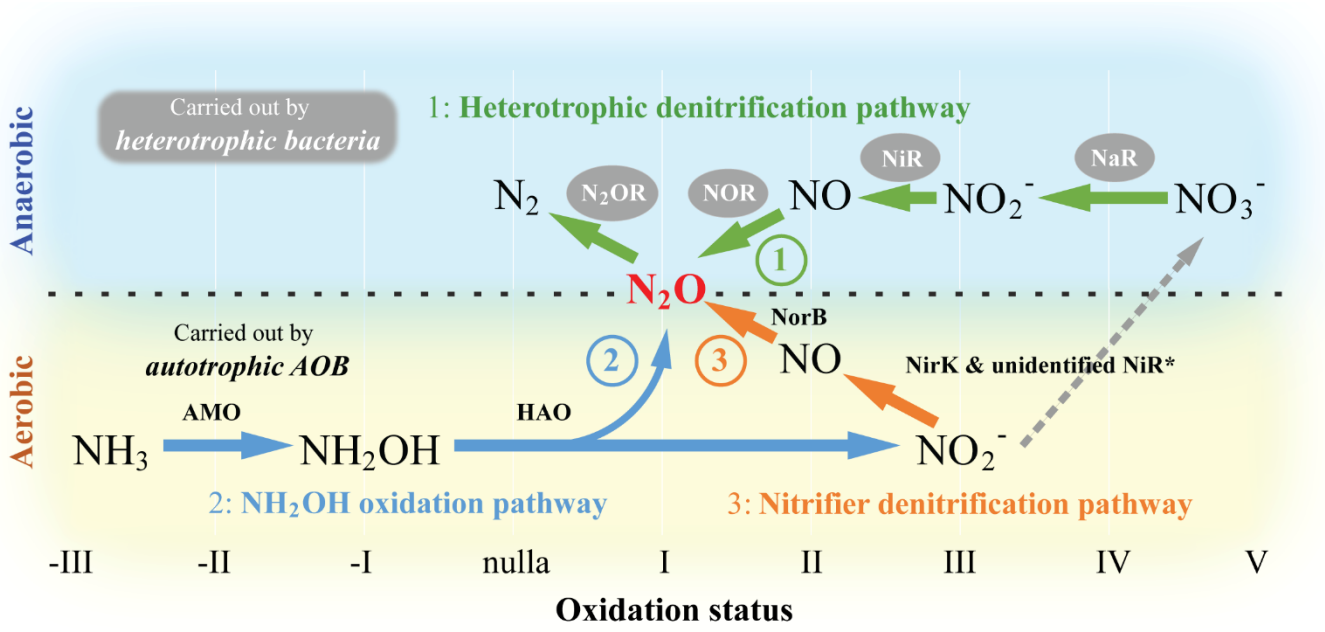
**Figure Legends**

**Figure 1** $N_2O$ produced in WWTS during biological nitrogen removal through three main microbial pathways: the heterotrophic denitrification pathway employed by heterotrophic denitrifiers, the $NH_2OH$ oxidation pathway and the nitrifier denitrification pathway employed by ammonia-oxidizing bacteria (AOB). AMO: ammonium monooxygenase; HAO: hydroxylamine oxidoreductase; NirK: copper-containing nitrite reductase; NorB: membrane-bound nitric oxide reductase; NaR: nitrate reductase; NiR: nitrite reductase; NOR: nitric oxide reductase; $N_2OR$: nitrous oxide reductase; *There is an unidentified nitrite reductase alternate to Nirk active in AOB (Kozlowski et al 2014).

**Figure 2** Simplified representation of the processes producing $N_2O$: (A) $NH_2OH$ oxidation and (B) NO reduction. For $NH_2OH$ oxidation, catalysed by HAO, nitroxyl (HNO) is formed as an intermediate. It then reacts with another $NH_2OH$ molecule to form hyponitrite (ONNO) which decomposes to $N_2O$. For NO reduction, two NO molecules, both bind with NOR, react to form hyponitrite (ONNO) which then engender $N_2O$. HAO: hydroxylamine oxidoreductase. NOR: nitric oxide reductase.

**Figure 3** Simplified representation of the processes controlling the positioning of the $^{14}N$ and $^{15}N$ atoms in $N_2O$ formed during: (A) $NH_2OH$ oxidation and (B) NO reduction. For $NH_2OH$ oxidation, the SP is largely controlled by the binding preference of NO to HAO, $^{14}N$ binds preferentially to HAO and the SP is positive. For NO reduction, the SP is expected to be close to zero because two NO molecultes simultaneously bond to the Fe centres of the NOR.

**Figure 4** Different interpretations based on different SP signature values. a, b, c, d refers to four kinds of SP values interpretations based on four sets of SP signature values adopted in different WWTP studies, see Table 4 for details.

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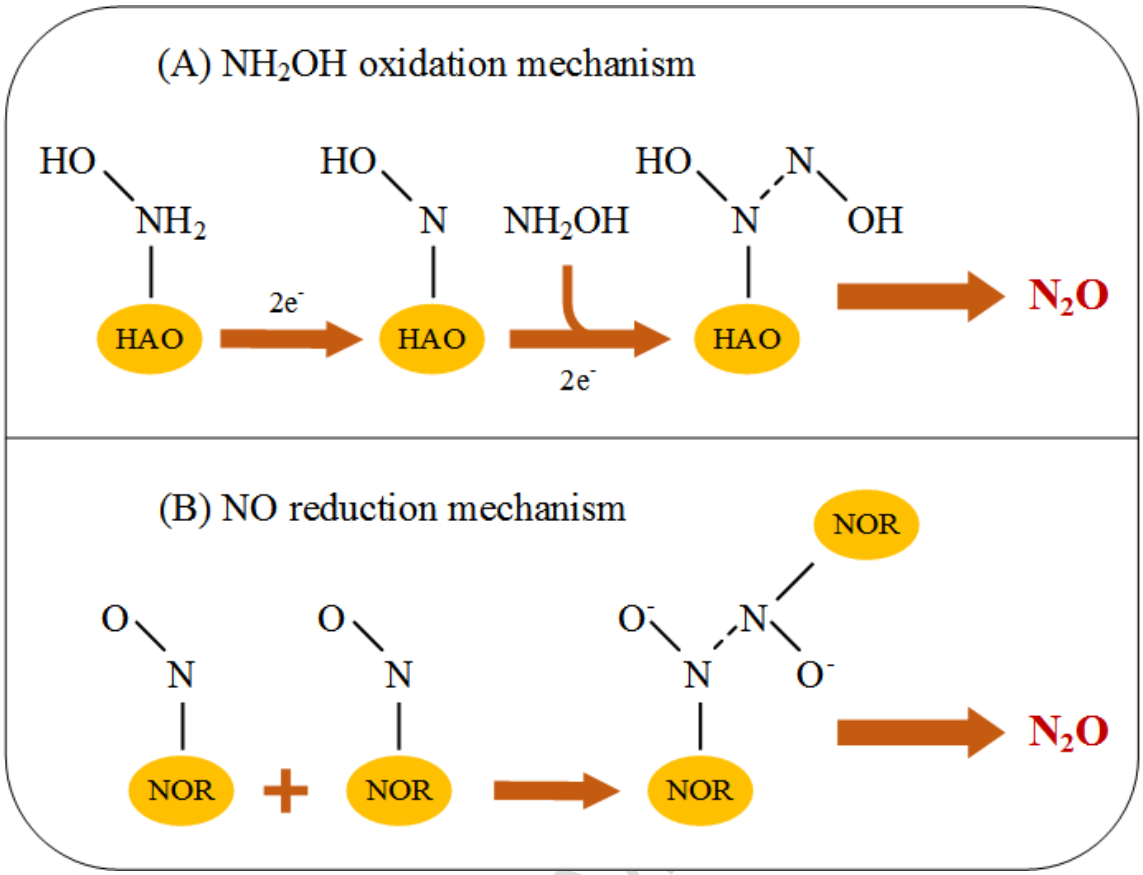


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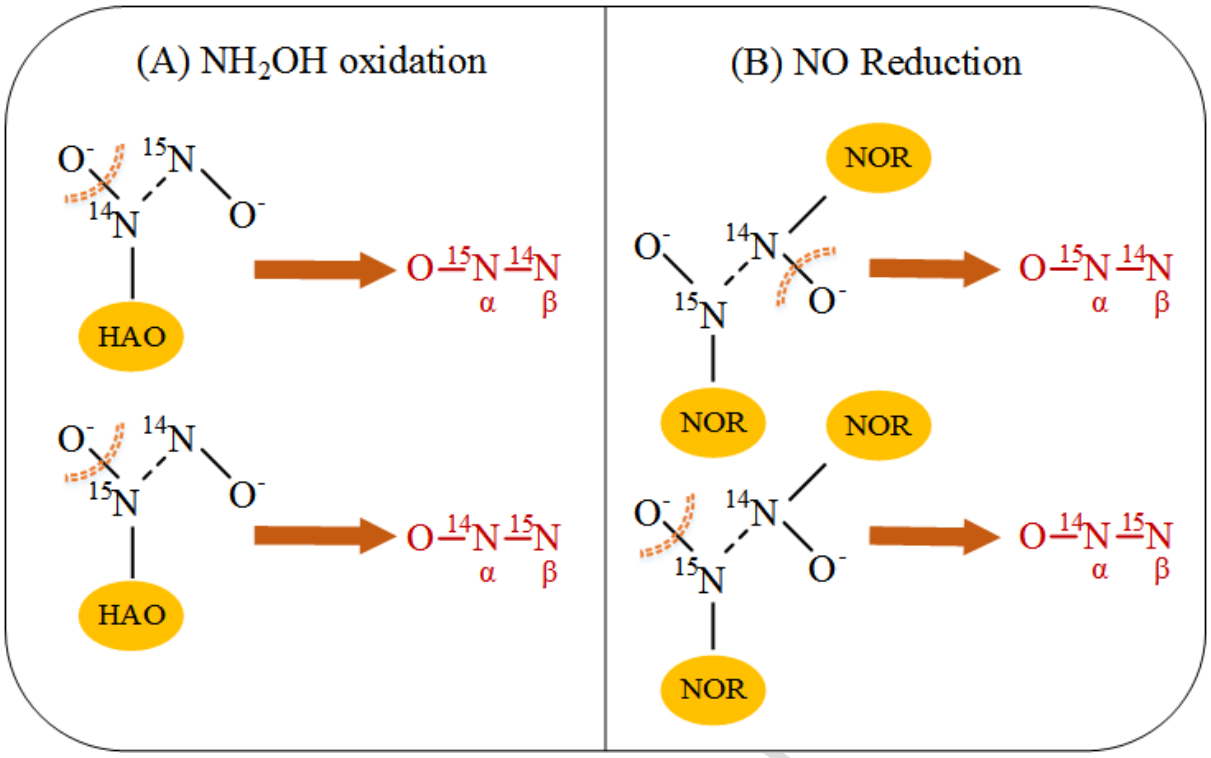


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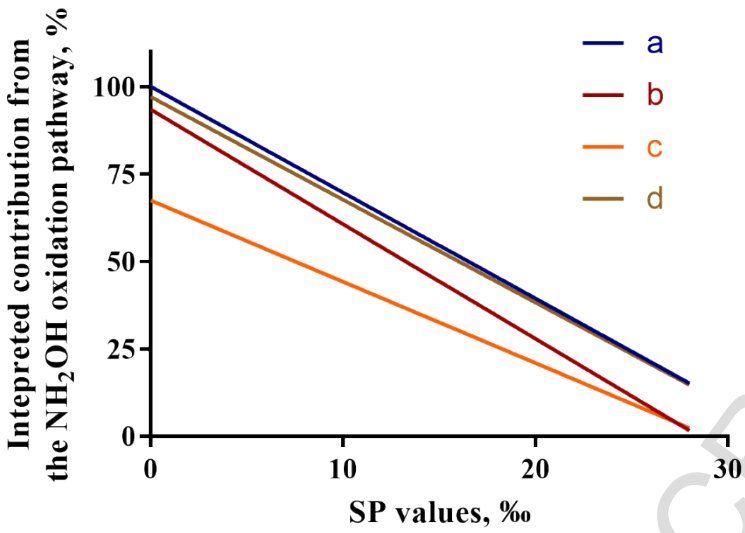


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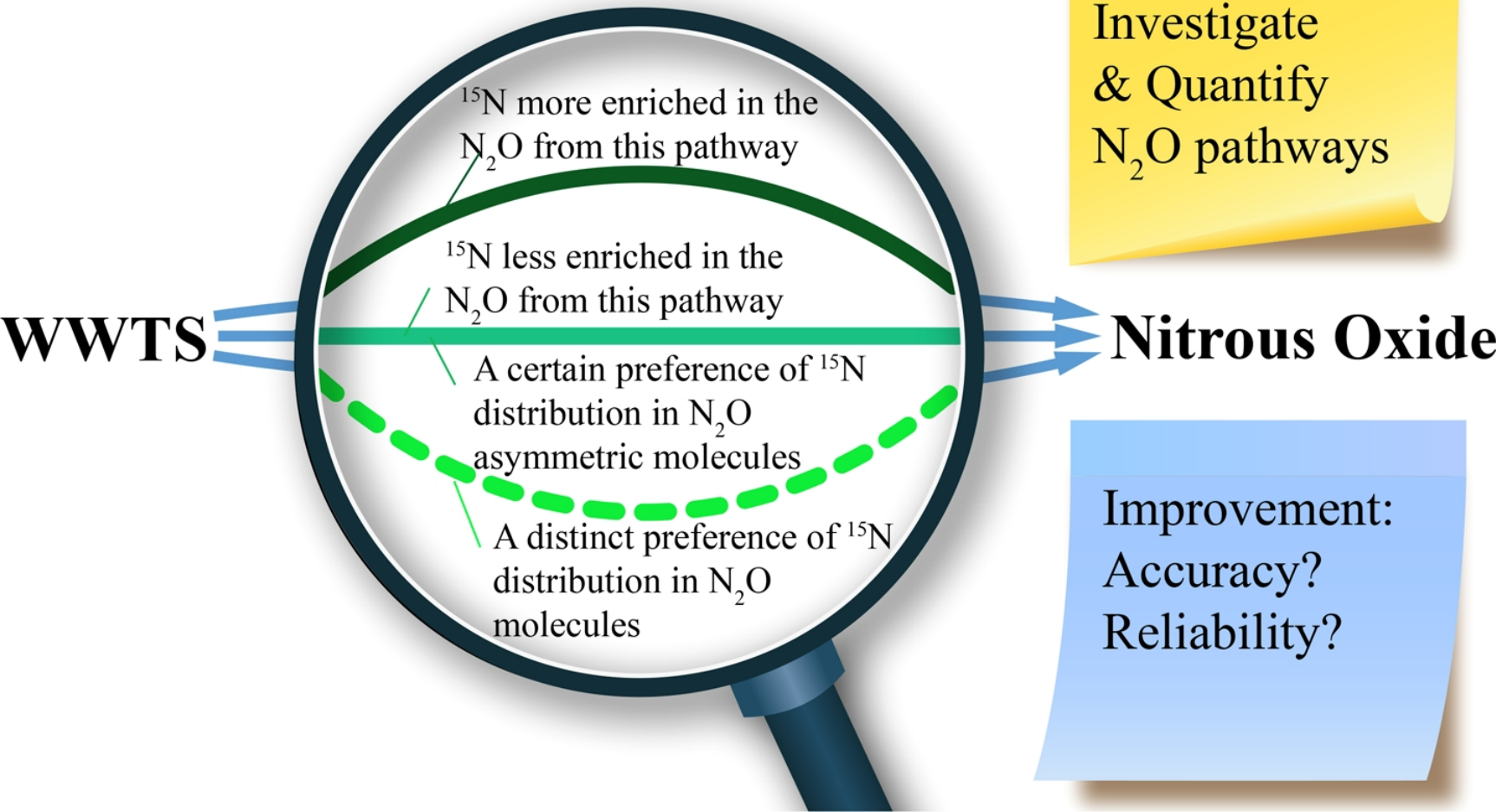
## Highlights

- A critical review of isotopic approaches quantifying $N_2O$ production pathways
- SP method is most promising while enrichment factor and tracer are of limited use
- Recognised universal SP signature values are required and crucial for SP method
- Opportunities are isotope methods in combination with other methods

# Isotope Technology

A powerful tool:
Investigate
& Quantify
$N_2O$ pathways




Improvement:
Accuracy?
Reliability?